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CARDIAC MYOFIBRIL ATPASE AND ITS REGULATION IN ENDURANCE EXERCISE AND DIABETES

by

ⓒ PATRICIA ELLEN MAYBANK

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
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A. N. Belcastro
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Date December 13, 1983

ABSTRACT

The purpose of this study was to investigate the responses of cardiac myofibril ATPase activity of endurance-swum, diabetic and control hearts under normal conditions and with increased inorganic phosphate (Pi) and/or decreased pH. Male Wistar rats (261 ± 7 g) were assigned to one of four groups: sedentary control (SC), trained control (TC), sedentary diabetic (SD) or trained diabetic (TD). Diabetes was induced by a single dose of streptozotocin (60 $mg \cdot kg^{-1}$). The endurance training groups underwent a progressive 8-week swimming program. Total swim time for TC was 29.94 ± 0.39 hours and 30.26 ± 0.34 hours for TD ($p > 0.05$). Maximally-activated (10 μM free Ca^{2+}) and basal, Mg^{2+}-stimulated (5 mM EGTA) myofibril ATPase activities were assayed with 0, 0.10 and 1.0 mM Pi at pH 7.0 and 6.5. Anthropometric results revealed greatly reduced heart weights (HW) and body weights (BW) in SD and TD versus their respective controls ($p < 0.001$). Endurance swimming resulted in 15.5% and 80% increases in the Ca^{2+}-activated and basal myofibril ATPase conditions respectively ($p = 0.05$). Diabetic hearts exhibited a profound depression in the Ca^{2+}-activated ATPase activity (SC: 0.084 ± 0.002 versus SD: 0.042 ± 0.005 $\mu mol\ Pi \cdot mg^{-1} \cdot min^{-1}$; $p < 0.001$) with a minimal response to training. In SC hearts, increasing Pi levels from 0 to 0.10 to 1.0 mM elicited a biphasic response of activation and inhibition ($p < 0.001$). In TC, this biphasic response was not evident; rather activation at 0.10 mM Pi was observed ($p = 0.05$) with little decline at 1.0 mM Pi ($p > 0.05$). Ca^{2+}-activated ATPase activity of SD increased from 0 to 0.10 mM Pi (0.042 ± 0.005 to 0.055 ± 0.005 $\mu mol\ Pi \cdot mg^{-1} \cdot min^{-1}$; $p = 0.05$) with negligible changes at 1.0 mM Pi ($p > 0.05$). TD hearts revealed a biphasic response typical of SC hearts. Acidosis (pH 6.5) resulted in an overall depression of Ca^{2+}-activated myofibril ATPase activities ($p < 0.001$). Lowered pH restored the biphasic response of TC to increasing Pi ($p < 0.001$). This was not observed in other groups ($p > 0.05$). The results of this research indicate that endurance swimming enhances myofibril ATPase activity of normal hearts but cannot reverse the depressive effects of diabetes. Decreased sensitivity to increasing Pi in TC at pH 7.0 may represent an adaptation allowing increased cross-bridge cycling rates and ATPase activity with maintenance of tension despite accumulating Pi. This adaptation appears to be pH-sensitive where acidosis abolishes the training response. The normalized pattern of ATPase response to Pi in TD suggests a role for endurance swimming in normalizing some aspects of ionic regulation of myofibril ATPase activity with diabetes despite continued depression of overall activity.

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CHAPTER I

INTRODUCTION

The capacity to physiologically adapt to repeated exercise stimuli is well-known. With regard to parameters of the cardiovascular system, chronic endurance exercise has been shown to elicit increases in the capillarization and oxygen uptake of skeletal muscles (Holloszy, 1976). Adaptations of central cardiovascular parameters are reflected by the increased cardiac output and stroke volume and bradycardia reported to occur in both humans and laboratory animals (Scheuer and Tipton, 1977). Enhanced functional performance of the heart has been demonstrated with endurance swimming and endurance running programs. Endurance-run dog and rat hearts have shown significant improvements in cardiac output and stroke volume (Barnard et al. 1980; Dowell et al., 1977; Schaible and Scheuer, 1979) although one running study does not agree (Fuller and Nutter, 1981). A greater enhancement of these variables over control hearts was further evidenced at elevated preloads following an endurance swimming program (Bersohn and Scheuer, 1977; Giusti et al., 1978; Schaible and Scheuer, 1979). Additional evidence indicates that swimming leads to enhanced left ventricular systolic pressure development and to a greater peak pressure (Hepp et al., 1974; Bersohn and Scheuer, 1977; Schaible and Scheuer, 1979). Increased maximum rate of rise of left ventricular pressure (max + dP/dt) also has been reported to accompany both chronic swimming and running exercise, this effect becoming more pronounced at elevated preloads (Penparkgul and Scheuer, 1970; Bersohn and Scheuer, 1977; Barnard et al., 1980). Stroke work and/or maximum power calculated from the product of pressure and flow indicate an improved pumping capacity of endurance-trained hearts (Bersohn and

Scheuer, 1977; Schaible and Scheuer, 1979). This improvement in pumping capacity in the absence of true hypertrophy has been attributed to increases in circumferential fiber shortening, contractile element shortening and in maximum rate of shortening (Schaible and Scheuer, 1979; Barnard et al., 1980; Schaible et al., 1981).

The apparent increase in cardiac contractility has been further evaluated to ascertain whether the contractility could be explained by the Frank-Starling mechanism due to increased end-diastolic volume (EDV) and increased ventricular compliance. In the absence of cardiac hypertrophy increased contractility was observed in the absence of altered EDV and EDP (ED pressure) following endurance swimming (Schaible et al., 1981; Schaible and Scheuer, 1979). One study which documented a 17% increase in the cardiac mass of endurance-swum female rats, did report a rightward shift in the EDV-EDP curve. Since EDV was greater for any given EDP the results were interpreted as an increase in cardiac efficiency (Hepp et al., 1974). The altered volume-pressure relationship with no accompanying increase in diastolic load on the ventricular wall suggests that a mechanical adaptation to training which involves the Starling mechanism is also present.

An adaptation to endurance swimming considered to be of major importance is that of increased maximal rates of cardiac relaxation (max - dP/dt) (Bersohn and Scheuer, 1977; Giusti et al., 1978; Schaible and Scheuer, 1979). This adaptation could enhance performance by permitting a greater diastolic filling time, thereby enhancing systolic performance in the face of increased heart rates as occurs with exercise.

The mechanisms underlying the enhanced cardiac contractility with training appears to involve biochemical events at the level of the sarcolemma, sarcoplasmic reticulum and myofibrils. A relationship between the functional and biochemical parameters of the myocardium has been established. Hamrell and Low (1978) found a 0.95 correlation between the myosin ATPase activity and the mechanical maximal unloaded shortening velocity (V_{max}) of rabbit ventricular muscle while Carey et al. (1979) observed a similar relationship in the cat. At submaximal levels Ca^{2+}-dependent tension generation and Ca^{2+}-activated ATPase activities have been found to parallel each other (Herzig and Ruegg, 1980; Fabiato and Fabiato, 1978). Based on this evidence researchers have considered adaptation of myofibril ATPase activity as a potential and logical, though not exclusive, mechanism by contributing to altered cardiac contractility.

A number of endurance swimming studies have substantiated this association. Elevations in cardiac myosin and actomyosin ATPase activities (Bhan and Scheuer, 1972 and 1975; Malhotra et al., 1976; Giusti et al., 1978; Penparkgul et al., 1978) and in cardiac myofibril ATPase activity (Rupp, 1981) have been reported (Table 1). Following 8 to 10 week training periods, deconditioning revealed a decline back to sedentary levels of myosin and actomyosin ATPase activities (Malhotra et al., 1976; Giusti et al., 1978). Moreover, the training and detraining alterations in actomyosin ATPase activity were parallelled qualitatively by alterations in cardiac output, coronary flow, myocardial O_2 consumption and maximal negative dP/dt (Giusti et al., 1978). Thus, biochemical parameters change in response to physiological overload.

Similarly, in pathological states such as diabetes mellitus functional disturbances in the heart have been traced to alterations in biochemical parameters. The decline in myocardial performance in diabetic hearts was previously considered to be of a vascular origin relating to alterosclerotic plaque formation (Ledet et al., 1979;

TABLE 1

SUMMARY OF ENDURANCE EXERCISE - CARDIAC ATPASE RESEARCH

Authors	Year	Animal Model	Exercise	ATPase	Results
Bhan, A.K. & Scheuer, J.	1972	male Wistar rats (200 g)	Swimming: 90 min/day; 4-6-8 wks (2 x 75 min/day) 8 wks	actomyosin: Ca^{2+}-activated & Mg^{2+}-activated	↑ Ca^{2+} ATPase (6 wks +; $p < 0.05$) ↑ Mg^{2+} ATPase (8 wks; $p < 0.01$) ATPase in 150 min/day sig. > 90 min/day
Bhan, A.K. & Scheuer, J.	1975	male Wistar rats (200 g)	Swimming: 90 min/day > 8 wks 2.75 mm/day	myosin Ca^{2+}-ATPase HMM Ca^{2+}-ATPase	90 min/day: 17% ↑ in myosin Ca^{2+}-ATPase 150 min/day: 30 ↑ ATPase HMM Ca^{2+}-ATPase ↑ ($p < 0.05$)
Baldwin, K.M. et al.	1975	male Wistar rats (100 g)	Running: 18-24 wks 1.2 mph/15% grade 2 h/day	actomyosin: Mg^{2+} and Ca^{2+} activated heart, soleus, red & white vastus	No sig. change in cardiac ATPase at 10 wks Mg^{++}-ATPase: $\left.\begin{matrix}0.325 \pm 0.025 \\ 0.335 \pm 0.025\end{matrix}\right.$ $\begin{pmatrix}S \\ T\end{pmatrix}$ vs $\mu mol\ Pi \cdot mg^{-1} \cdot min^{-1}$ 20% ↑ in red vastus ATPase 20% ↑ in soleus

Authors	Year	Animal Model	Exercise	ATPase	Results
Baldwin, K.M. et al.	1977	female Wistar rats (120 g)	Running: Steady-state: 1 hr/1.6 km/25% gr 10 wks Interval: 9 wks 3.2-4 km/hr; 6% gr 3,2,1-min intervals (90 min)	myofibrils: Mg^{2+} & Ca^{2+} activated (cardiac & skeletal)	transient ↑ (to 4 wks) in Mg^{2+}-ATPase in cardiac, then N.S. diff. Ca^{2+}-ATPase: N.S. diff w/Steady-state protocol Interval: 15% ↑ in Mg^{2+}-ATPase (cardiac) after 9 wks ($p < 0.05$) similarly for Ca^{2+}-ATPase
Dowell, R.T. et al.	1977	adult mongrel dogs; M & F	Running: 75 min/day x 5 days 8-10 wks	myofibril ATPase	No change: S: 0.199 ± 0.012 T: 0.194 ∓ 0.012 $\mu mol\ Pi \cdot mg^{-1} \cdot min^{-1}$
Giusti, R. et al.	1978	male Wistar rats	Swimming: 2 x 75 min; 5 days/wk; 8 wks deconditioning period - 2 wks in one group	actomyosin ATPase	ATPase sig. > w/cond. 0.60 ± 0.02 vs 0.68 ∓ 0.01 $\mu mol\ Pi \cdot mg^{-1} \cdot min^{-1}$ ($p < \bar{0}.001$) Decond. 2 wks: ATPase returned to sed. levels
Hearn, G.R. & Gollnick, P.D.	1961	male Sprague-Dawley rats	Swimming: 30 min/day; 7 days/wk; 5 wks	not specified	ATPase act. of ventricles from cond. rats > sed. rats ($p < 0.01$)

Authors	Year	Animal Model	Exercise	ATPase	Results
Malhotra, A. et al.	1976	male Wistar rats (200-240 g)	Swimming: 2 x 75 min/day; 5 days/wk 8 wks to 16 wks 8 wks + 8 wks decond. 8 wks + 8 wks at 45 min/day	actomyosin: Ca^{2+}-stimulated ATPase	17 to 26% ↑ in ATPase act. after 8 wks ($p < 0.01$) Deconditioned: ATPase ↓ to sed. levels by 13 days Reduced Intensity: ATPase ↓ to drf. ny 30 days 16 wks tr. = 8 wks
Medugorac, I.	1975	rats	Swimming: 100-120 hr; 6-10 wks	actomyosin & myosin Ca^{2+}-ATPase	Ca^{2+}-ATPase act. of actomyosin & myosin sig. > after conditioning
Penparkgul, S. et al.	1980	male Wistar rats (180-200 g)	Running: A. 2 - 75 min/day 5 days/wk 8 wks of 20 m/min; 8% gr. B. 120 min/day; 5 days/wk 12 wks at 31 m/min; 8% gr. w/30 s. sprints	actomyosin & myosin Ca^{2+}-ATPase Actin-act. Mg^{2+}-ATPase S.R. ATPase	Protocol A: 8 wks T > S actomyosin ATPase $p < 0.001$ ($0.585 \pm .010$ vs 0.531 ± 0.011) $\mu mol\ Pi \cdot mg^{-1} \cdot min^{-1}$ Protocol B: 12 wks N.S. diff T vs S Pair weighted actomyosin ATPase sig ↓ vs T & S Actin-activation 7% > in T SR-ATPase T = S

Authors	Year	Animal Model	Exercise	ATPase	Results
Carey, R.A. et al.	1979	Dog	Running: 5 days/wk; 10 wks. Maximum: 75 min/day; up to 9 miles/hr; up to 20% grade	myosin	ATPase V_{max} not altered S: 1.74 ± 0.20 μmol $Pi \cdot mg^{-1} \cdot min^{-1}$ T: 1.65 ± 0.21 μmol $Pi \cdot mg^{-1} \cdot min^{-1}$
Penparkgul, S. et al.	1979	female rats	moderate endurance Swimming	actomyosin: Ca^{2+}-activated SR ATPase: Mg^{2+}- & Ca^{2+}-Mg^{2+} activities	Hypertrophy (24%) ATPase ↑ 18% (SW. vs C) (0.84 ± 0.10 vs 0.71 ± 0.01) SR-ATPases: No change
Rupp, H.	1981	male Wistar rats	Swimming: start: 30 min finish: 180 min/day 7 days/week 4 weeks	myofibril Ca^{2+}-activated; myosin isoenzymes	ATPase ↑ 10% (T vs C: $p < 0.001$)
Resink, T.J. et al.	1981	male Long-Evans rats	Running: by week 5: 2 hrs/day 15° incline 0.8-1.0 mi/hr 9 weeks	Ca^{2+}-sensitive actomyosin; myosin a) Ca^{2+} b) K^+-EDTA	Ca^{2+}/Mg^{2+} actomyosin: C = .162 μmol $Pi \cdot mg^{-1} \cdot min^{-1}$ T = .156 μmol $Pi \cdot mg^{-1} \cdot min^{-1}$ No sig. difference Ca^{2+}-myosin ATPase: C = .279 μmol $Pi \cdot mg^{-1} \cdot min^{-1}$ T = .322 μmol $Pi \cdot mg^{-1} \cdot min^{-1}$ $p < 0.05$ K^+-EDTA myosin ATPase: No change

Authors	Year	Animal Model	Exercise	ATPase	Results
Tibbits, G. et al.	1978	male Sprague-Dawley rats	Running: 5 days/wk 11 weeks final level: 60 min/day, 1.8 km/hr, 10% incline	myofibril: Mg^{2+}-stimulated Ca^{2+}-activated	No sig. alterations Mg^{2+}-stimulated ATPase: $T = 0.308 \pm 0.012$ $S = 0.324 \pm 0.006$ $\mu mol\ Pi \cdot mg^{-1} \cdot min^{-1}$ Ca^{2+}-activated: $T = 0.447 \pm 0.015$ $S = 0.473 \pm 0.011$ $\mu mol\ Pi \cdot mg^{-1} \cdot min^{-1}$
Wilkerson, J.E. & Evonuk, E.	1971	male Sprague-Dawley rats (367 g)	Swimming: 3 groups: 1. 30 min on alternate days; 10 weeks. 2. to exhaustion with 5% B.W.; alternate days; 6 weeks 3. Same as 2; 10 weeks	myosin- Ca^{2+}-activated gastrocnemius myosin, Ca^{2+}-ATPase	cardiac myosin Ca^{2+}-ATPase: ↑ ($p < 0.01$) on all three protocols $T = 43.50 \pm 1.74$ $C = 29.54 \pm 1.77$ $\mu mol\ Pi \cdot mg^{-1} \cdot min^{-1}$ T-3 vs S Gastrocnemius-myosin Ca^{2+}-ATPase: ↑ in group 2 & 3 ($p < 0.01$)

S = Sedentary

T = Trained

Sinclair-Smith, 1979; Vihert et al., 1969). However, in recent years evidence has accumulated to suggest the existence of a myocardial disorder of non-vascular origin in diabetes (Strobeck et al., 1979). Investigations in humans (Hamby et al., 1974; Rubler et al., 1972; Regan et al., 1976; Ledet, 1976) and in experimental animal models of diabetes (Regan et al., 1974; Strobeck et al., 1979; Giacomelli and Werner, 1979; Fein et al., 1980; Dillman, 1980) have described multiple functional, biochemical and ultrastructural alterations in diabetic hearts in the absence of coronary artery involvement.

Hemodynamic studies on diabetic patients revealed pre-clinical, functional alterations which could preceed full-scale heart failure (Regan and Wiese, 1978). Altered systolic time intervals including a shortened left ventricular ejection fraction (LVET), a prolonged pre-ejection period (PEP), an increased ratio of PEP:LVET and a greater isovolumic contraction time were considered pre-clinical manifestations of heart failure in diabetic patients (Hamby et al., 1974; Ahmed et al., 1975; Zoneraich et al., 1977; Haider et al., 1978). An altered PEP:LVET ratio is indicative of reduced rate of rise of pressure and potential alteration of the myocardial force-velocity relationship (Zoneraich et al., 1977). One study on adult-onset diabetics (Type II) demonstrated altered left ventricular EDP:EDV ratios where EDV and stroke volume were depressed while afterload increments significantly increased filling pressures without compensation in stroke volumes (Regan et al., 1977). Post-mortem evidence has revealed accumulation of interstitial glycoprotein material, collagen deposition, and enhanced triglyceride and cholesterol deposit all of which are suggestive of a diffuse extravascular abnormality in the diabetic heart. The glycoprotein accumulation could contribute to the decreased distensibility and increased pressure on the left ventricle observed during diastole (Regan et al., 1977).

Electron microscope examinations have further revealed an extensive pathomorphology in diabetic animal hearts. Spontaneously-diabetic mice (Giacomelli and Weiner, 1979) and streptozotocin-diabetic rats (Onishi et al., 1981) revealed degrees of endocardial fibrosis, basement membrane thickening, the appearance of lipid droplets and mitochondrial degeneration. Myocyte degeneration involving partial myofibrillolysis, irregularity of Z-lines, disrupted sarcomere organization and varying degrees of contraction in the later states of disease were documented (Giacomelli and Weiner, 1979).

As with physical training, the physiological alterations described appear to be partially a result of abnormalities in the biochemical indices of cardiac contractile behavior. Significant reductions in cardiac myofibril ATPase activity (Pierce and Dhalla, 1981), and in actomyosin ATPase activity (Malhotra et al., 1981) have been reported for streptozotocin-diabetic hearts as well as in the myosin ATPase activity of alloxan-diabetic hearts (Dillman, 1980). This effect included basal, Mg^{2+}-stimulated myofibril ATPase activity as well as the Ca^{2+}-activated state while no variation in the Ca^{2+}-dependence of the myofibrils was observed (Pierce and Dhalla, 1981). Impairment of the actomyosin and myosin Ca^{2+}-ATPase activities could be detected as early as 48 hours to one-week post-injection with significant declines reported at 2 and 4 weeks and persisting up to 8 weeks (Malhotra et al., 1981; Garber and Neely, 1981; Dillman, 1980). Actin-activation of the Mg^{2+}-stimulated ATPase was also reduced from an 11-fold activation in controls to an 8-fold activation in diabetic hearts (Malhotra, 1981).

The molecular changes possibly contributing to a modified ATPase activity have also been examined. The type or extent of both basal,

Mg^{2+}-stimulated and Ca^{2+}-activated myofibril ATPase responses to increasing KCl concentrations were reported to differ providing evidence of fundamental differences in protein structure of control and diabetic myofibrils (Pierce and Dhalla, 1981). Involvement of thiol groups on the myosin heavy chains has also been implicated. Modification of SH_1 and SH_2 groups elicited a much greater response of diabetic myosin Ca^{2+}-ATPase activity compared to controls (Malhotra et al., 1981). In addition ethylene glycol, which detects conformational changes at or near the myosin active site, elicited a differential response of diabetic and control basal ATPase (Pierce and Dhalla, 1981). Therefore a conformational change, possibly an unfolding, influences the reactivities of myosin thiol groups thought to participate in the ATPase reaction. A further alteration accompanying diabetes is a shift in myosin isoenzyme expression where the V_3 form with its inherently slower ATPase activity, assumes predominance over the faster normally-dominant V_1 form and the heterogenous V_2 (Dillman, 1980). Interestingly, insulin administration, following induction of diabetes, was shown to normalize the myosin isoenzyme pattern and myosin-ATPase activity (Dillman, 1980).

The depressed ATPase activities seem to be characteristic of a cardiomyopathic disorder. Similar alterations have been reported in non-diabetic cardiomypathic hamsters (Strobeck et al., 1979; Pang and Weglicki, 1980) which indicates the possibility of a primary cardiomyopathy associated with diabetes as opposed to being secondary to the disease state.

The observation that the myocardial functional and biochemical alterations associated with endurance training and diabetes mellitus are similar but directionally opposed leads to the suggestion that one stimulus may influence the response to the other. Therefore, a major thrust of this research was to investigate the possible ameliorative influence of endurance swimming on the cardiac myofibril ATPase activity of diabetic rats.

Myofibril ATPase is also subject to regulation by a number of ions and mechanisms which may dramatically alter its activity. Reduction of intracellular pH has been shown to reduce the Ca^{2+}-sensitivity of tension and myofibril ATPase activity in heart muscle as well as reducing maximal and basal activities (Fabiato and Fabiato, 1975[a]; Donaldson et al., 1981; Kentish and Nayler, 1979; Okabe and Hess, 1981). The metabolic irregularities which occur in diabetic hearts such as accumulation of metabolic intermediates (ketone bodies and amino acids) as well as reduced oxidative phosphorylation (Opie et al., 1979) could result in a greater H^+ concentration and subsequent acidotic-depression of ATPase. In addition, a regulatory role for the inorganic phosphate ion (PO_4^{3-}) has been postulated. Non-diabetic hearts have demonstrated decreased tension development and immediate stiffness despite increased myofibril ATPase activities in response to elevated phosphate ion concentrations in the millimolar range (Herzig et al., 1981; Solaro et al., 1980[a] and [b]). Accumulation of phosphate ions has been shown to accompany skeletal muscle fatigue (Kushmerick and Crow, 1982) therefore chronic activity may alter the response of cardiac muscle to this ion.

Since inorganic phosphate content and intracellular acidosis have been implicated in the depression of the contractile response (Jacobus et al., 1977; Donaldson et al., 1981; respectively) the role of these parameters in regulating the myofibril ATPase activity of cardiac muscle from diabetic and endurance-trained animals has also been investigated.

CHAPTER II

METHODOLOGY

1. Animal Care

Male Wistar rats (Woodlyn Farms) (N = 63) initially weighing 261 ± 7 grams were randomly assigned to one of four groups: sedendary control (SC; N = 13), trained control (TC; N = 15), sedentary diabetic (SD; N = 17) or trained diabetic (TD; N = 18). A greater number of animals was allotted to training and diabetic groups in order that a sufficient final N would be attained despite unsuccessful induction of diabetes from injection, sickness or death due to diabetes or drowning. The overall attrition rate from the above causes was 18.25%, while separately it was 41.9% in TC, 13.7% in SD, 20% in TD and 0 in SC. The final N used for biochemical and statistical analyses was 7 per group.

The animals were individually housed in wire mesh cages with a controlled environmental setting of 20 ± 1°C and 50 + 5% humidity and were maintained on a reverse day/night cycle of 12 hours (i.e., dark: 8:00 a.m. to 8:00 p.m.). Laboratory chow and water were provided ad libitum. Body weights were obtained once per week throughout the entire experiment. Both S and T groups were subjected to similar disruptions in cycle with respect to light, noise and change of location on the training days, the T groups being distinguished mainly by slightly more handling, water immersion and exercise.

2. Induction and Assessment of Diabetes (Details in Appendix B-1)

All SD and TD animals were made diabetic by a single injection of streptozotocin (60 mg/kg; Upjohn Co.) dissolved in citrate buffer (pH 4.5) and administered via the penile dorsal vein under mild ether anasthesia

(1-2 min). All non-diabetic animals received a comparable volume of citrate buffer under the same conditions.

Diabetes was assessed by plasma and urine glucose analysis using the oxygen rate method of Kadish (1965) adapted for the Beckman Glucose Analyzer 2. Non-fasting, micro-blood samples were collected from the tail vein of conscious animals into non-heparanized hematocrit tubes one week following injection and at 4 and 8 weeks of the training program. Samples were centrifuged at 1000 x g for 10 minutes, plasma transferred to clean tubes and frozen at -20°C until analysis. Plasma glucose levels exceeding 400 $mg \cdot dl^{-1}$ were considered adequate indication of the diabetic state. Urine samples were obtained at 4 and 8 weeks from diabetic and non-diabetic animals placed overnight in metabolic collection cages and fasted. Urine samples were then centrifuged at 1000 x g for 5 minutes, the supernatant removed and frozen at -20°C until analysis.

3. Training Program

The endurance training program consisted of progressively-increased bouts of swimming 4 days per week for 8 weeks (Table 2). The animals' tails were weighed with 2% of each animal's body weight which was adjusted weekly. Water temperature was maintained between 32° and 35°C during each session. Five to six animals swam together in rubber chambers of height 66 cm, diameter 54 cm, water depth 52 cm and surface area 1808.6 cm^2.

Animals were sacrificed 48-72 hours after completion of the program by stunning and decapitation.

4. Tissue Sampling

Following sacrifice and exsanguination, the hearts were quickly excised, the atria and greater vessels trimmed away and the ventricles

TABLE 2

SWIM-TRAINING PROGRAM

Week	Duration (minutes)					
	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday/ Sunday
1	20	25	Rest	30	30	Rest
2	35	35		40	40	
3	45	45		50	50	
4	50	55		55	60	
5	60	65		65	65	
6	70	70		75	75	
7	75	80		80	85	
8	85	85		90	90	

blotted and rinsed in cold saline solution. Ventricles were then weighed, frozen in isopentane pre-cooled with liquid nitrogen and stored at -80°C for analysis.

5. Biochemical Analysis
(Details in Appendix B)

Myofibril isolation followed the procedures of Perry and Corsi (1958) with the modifications of Solaro et al. (1971). Whole ventricles were homogenized with a Polytron PT-10 in 20 volumes of cold borate-KCl-EDTA buffer (pH 7.1: 39 mM Na-borate, 25 mM KCl and 5 mM EDTA). Following centrifugation at 1000 x g for 10 minutes the supernatant was discarded, the pellet resuspended in the relaxing buffer and recentrifuged. The pellet of relaxed myofibrils was then double-rinsed and similarly centrifuged in 20 volumes of cold borate-KCl buffer (pH 7.1: 39 mM

Na-borate, 25 mM KCl). Double-washing and centrifugation procedures in a detergent-Na-Azide buffer (pH 7.4: 100 mM KCl, 50 mM Tris base, 1.0% Triton-X-100 and 10 mM Na-Azide) solubolized and removed contaminating mitochondrial, sarcolemmal and sarcoplasmic reticular membranes (Solaro et al., 1971) and inhibited mitochondrial membrane-bound ATPases. Previously, samples run with and without Na-Azide have shown < 5% difference with regard to ATPase activity indicating removal of contaminating ATPases (Belcastro et al., 1982). The pellet was then resuspended in a suspension medium (pH 7.4: 150 mM KCl and 50 mM Tris base) and centrifuged as before. The final pellet of myofibrils, free of contaminating membranes and ATPases and in the relaxed state, was then resuspended in a small volume of the suspension medium (3 to 6 mls) and a 0.10 ml aliquot taken for protein determination by the method of Lowry et al., (1951). Myofibril protein concentration was then adjusted to 2 mg·ml^{-1}.

Determination of myofibril ATPase activity was carried out according to Goodno et al. (1978) as modified by Belcastro et al. (1982). Both the Ca^{2+}-activated (10 mM free Ca^{2+}) and the basal, Mg^{2+}-stimulated (5 mM EGTA) ATPase activities were assayed under the conditions shown in Table 3 in duplicate. In the assay procedure, to the reaction mixture (100 mM KCl, 4 mM Tris base and 2 mM $MgCl_2$) was added the Ca^{2+}-EGTA buffer, the suspension medium or inorganic phosphate (Pi) solution, the myofibril protein (0.5 mg·ml^{-1} final concentration) and 12% TCA to the blanks. Following a 5 minute pre-incubation at 30°C in a shaker water bath, the reaction was started with addition of 5 mM $Mg{\cdot}ATP^{2-}$ and incubated exactly 5 minutes at 30°C in the water bath with periodic light vortexing to prevent protein sedimentation. The reaction was stopped by addition of cold 12% TCA and all tubes were placed immediately in ice

TABLE 3

EXPERIMENTAL DESIGN

Grouping Variables		In Vitro Biochemical Conditions											
Training Level	Diabetic Level	pH 7.0						pH 6.5					
		0 mM Pi*		0.10 mM Pi		1.0 mM Pi		0 mM Pi		0.10 mM Pi		1.0 mM Pi	
Sedendary	Control		X		X		X		X		X		X
	Diabetic		X		X		X		X		X		X
Trained	Control		X		X		X		X		X		X
	Diabetic		X		X		X		X		X		X

* Pi = inorganic phosphate (KH_2PO_4)

'X' denotes a blank tube accompanying each reaction tube. In 'X', reaction inhibited with 12% TCA.

for 10 minutes. The liberated inorganic phosphate from the ATPase reaction was determined in the protein-free supernatant following a 10 minute centrifugation at 1000 x g (Taussky and Short, 1953). Optical densities were read on a Pye-Unicam SP-1800 Ultraviolet Spectrophotometer. ATPase activity was expressed as micromoles Pi per milligram protein per minute. The 10 μM Ca^{2+} concentration in the Ca^{2+}-activated state was calculated according to the binding constants of Solaro and Shiner (1976) which account for the differing Ca^{2+}-EGTA affinities at pH 7.0 and pH 6.5. Basal, Mg^{2+}-stimulated ATPase was obtained with addition of 5 mM EGTA. The inorganic phosphate (Pi) solutions contained either 0.10 mM or 1.0 mM KH_2PO_4. The reaction medium, phosphate solutions, suspension medium, Ca^{2+}-buffers and $Mg \cdot ATP^{2-}$ were duplicated, each at pH 7.0 and at pH 6.5 (30°C) for the experimental protocol (Table 3).

6. Statistical Analysis

Plasma glucose values in SC versus SD and in SD versus TD, over the three time points, were analyzed using a one-way analysis of variance (ANOVA) with repeated measures on time. Ventricular weight data, body weight data and HW:BW data were each analyzed through a two-way ANOVA (2 x 2). Myofibril ATPase activities for Ca^{2+}-activated and basal conditions were separately analyzed through a four-way ANOVA with repeated measures on the last two factors (2 x 2 x 2 x 3). A posteriori comparisions were made by the Tukey method and significant differences at the 95% confidence interval ($p < 0.05$) or better were accepted.

CHAPTER III

RESULTS

Non-fasting plasma glucose values obtained from SC at 1, 4 and 8 weeks were comparable (Table 4). An elevation in plasma glucose levels of SD over those of SC was observed at all time points (i.e., week 8: SD = 680.8 ± 33.5 $mg \cdot dl^{-1}$ versus SC = 121.8 ± 7.8 $mg \cdot dl^{-1}$, $p < 0.001$) ($\overline{X}$ ± S.E.M.). Similarly, TD values were markedly increased over SC throughout the duration of the experiment (Table 4). The plasma glucose values for both SD and TD were similar over time ($p > 0.05$). With regard to fasting urine glucose levels, the SC value at week 8 was 200.6 ± 59.6 $mg \cdot dl^{-1}$ compared to 8366.6 ± 1459 $mg \cdot dl^{-1}$ and 6427.7 ± 1427.6 $mg \cdot dl^{-1}$ for SD and TD respectively.

The mean total swim times upon completion of the endurance training program were 29.94 ± 0.39 min for TC and 30.26 ± 0.34 min for TD. All training animals were capable of swimming continually for 90 minutes during the final session.

The ventricular weights of S and T animals were similar in both the control and diabetic conditions (Table 5). Diabetes elicited a pronounced reduction in ventricular tissue weight relative to the controls in both S and T animals ($p < 0.001$) (Table 5). With regard to body weight, no alterations due to endurance training were observed in either control or diabetic animals (Table 5). However, diabetes induced large reductions in weight gain as reflected by lower end BW in both SD and TD relative to SC and SD respectively ($p < 0.001$) (Table 5). The ratio of ventricular weight to body weight (HW:BW) revealed no effect of endurance training in control and diabetic animals. An increase in the HW:BW ratio of SD animals

TABLE 4

PLASMA GLUCOSE DATA*

Group†	Plasma Glucose (mg·dl^{-1})		
	Week 1	Week 4	Week 8
Control	129.6 ± 3.0	125.3 ± 3.4	121.8 ± 7.8
Sedentary Diabetic	784.3 ± 95.4 [a]	779.0 ± 58.2 [a]	680.8 ± 33.5 [a]
Trained Diabetic	665.2 ± 61.9	630.6 ± 59.0	680.3 ± 69.0

* values expressed as $\bar{X} \pm$ S.E.M.

† no significant differences between groups or across time

[a] = SD versus control, $p < 0.001$

TABLE 5

HEART WEIGHT-BODY WEIGHT DATA*

Group	Heart‡ Wt. (mg)	Body Wt. (g)	HW:BW
Sedentary Control	1145.5 ± 31.0	468.7 ± 8.4	2.47 ± 0.10
Trained Control	1126.0 ± 18.9	419.3 ± 8.4	2.68 ± 0.08
Sedentary Diabetic	852.8 ± 38.7 [a]	291.3 ± 23.9 [a]	2.98 ± 0.12 [c]
Trained Diabetic	870.7 ± 48.9 [b]	301.7 ± 16.4 [b]	2.88 ± 0.06

* values expressed as $\bar{X} \pm$ S.E.M.

‡ heart weight refers to ventricular weight

[a] = SC versus SD, $p < 0.001$

[b] = TC versus TD, $p < 0.001$

[c] = SC versus SD, $p < 0.05$

relative to SC was observed ($p < 0.05$) (Table 5) while in TD animals this elevation was noted but was not significant relative to TD. Examination of the contribution of the two HW:BW ratio components to the overall increased ratio in diabetes exposed a greater proportionate decrease in body weight compared to heart weight relative to control values in both SD and TD groups (SD: 25% HW versus 38% BW; TD: 22.7% HW versus 28.1% BW).

Myofibril protein yields from the isolation procedures expressed as milligrams protein per gram wet tissue weight were comparable across all 4 groups (SC: 35.12 ± 0.89; TC: 35.08 ± 0.83; SD: 33.53 ± 0.78; TD: 36.50 ± 1.78 $mg \cdot g^{-1}$).

The maximally-activated (10 μM free Ca^{2+}) myofibril ATPase activity of SD was strikingly depressed relative to that of SC ($p < 0.001$) while the two groups displayed similar basal, Mg^{2+}-stimulated ATPase activities at normal pH (Figure 1). The basal activities corresponded to 11.9% and 40.5% of SC and SD maximal ATPase values, respectively. Maximal activation at pH 6.5 elicited an elevation in SC over SD ($p < 0.05$) while basal activities at pH 6.5 were comparable ($p > 0.05$) (Figure 2). Expressed as percent of maximally-activated ATPase activity, the basal SC and SD activities were 40.6% and 52.9%, respectively.

The Ca^{2+}-activated myofibril ATPase of the SC group at pH 7.0 demonstrated a distinct biphasic response to increasing concentrations of inorganic phosphate (Pi) where activation was observed at 0.10 mM Pi (0.084 ± 0.002 to 0.112 ± 0.005 $\mu mol\ Pi \cdot mg^{-1} \cdot min^{-1}$; $p < 0.001$) followed by inhibition and return to normal levels at 1.0 mM Pi ($p < 0.001$) (Figures 3a and 3b). This pattern became somewhat flattened in the SD group where some activation (30.9% above normal) but little inhibition was observed. At all Pi concentrations the SC ATPase activities

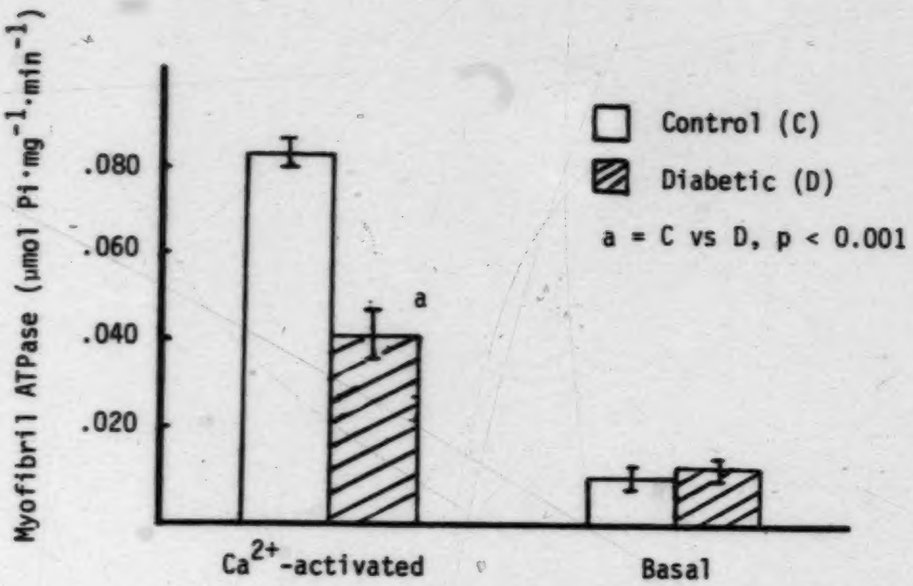

Figure 1. Ca^{2+}-activated and basal myofibril ATPase activities in control and diabetic sedentary hearts at pH 7.0

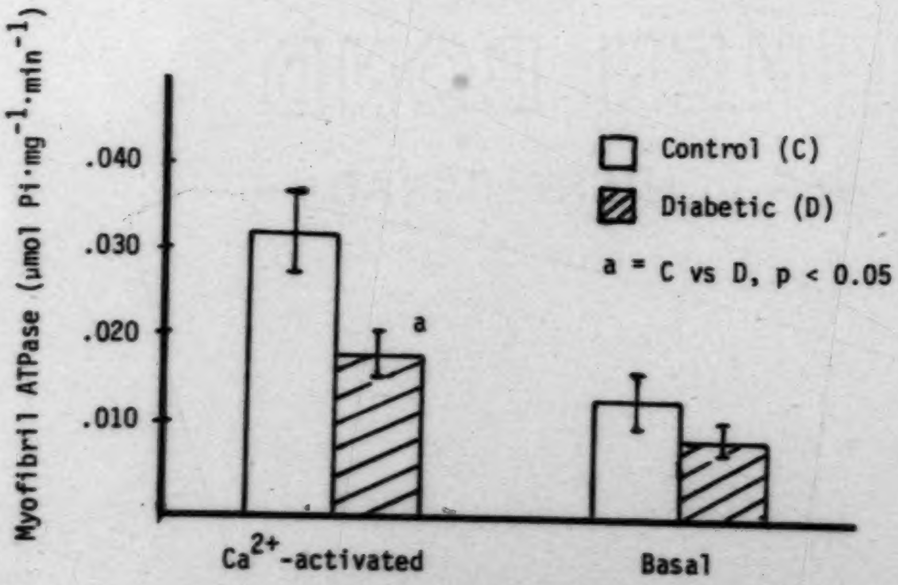

Figure 2. Ca^{2+}-activated and basal myofibril ATPase activities in control and diabetic sedentary hearts at pH 6.5

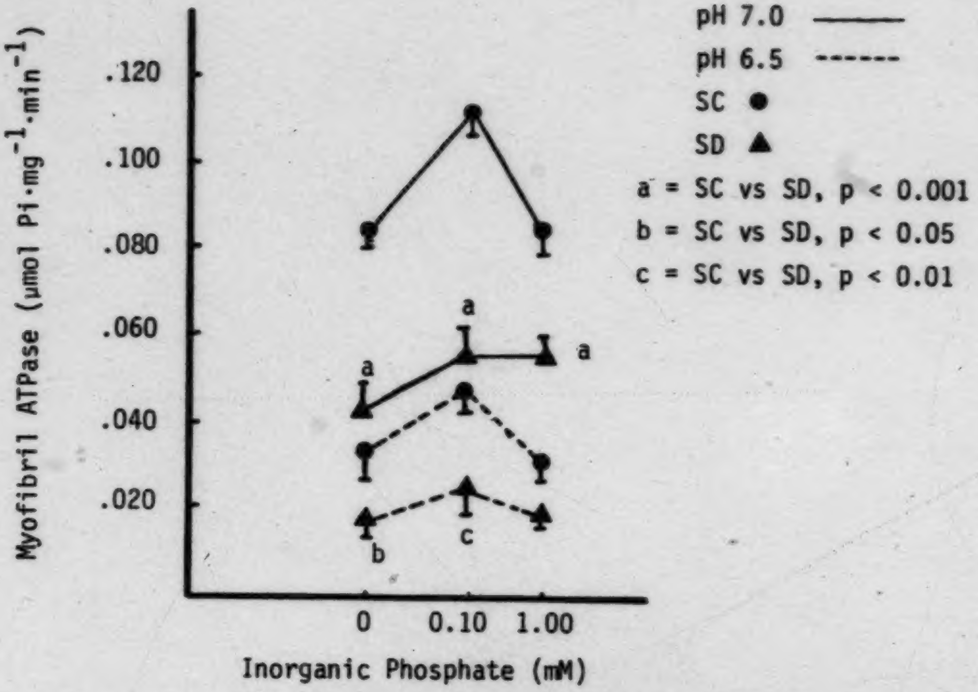

Figure 3a. Inorganic phosphate influence on the Ca^{2+}-activated myofibril ATPase activity on sedentary hearts

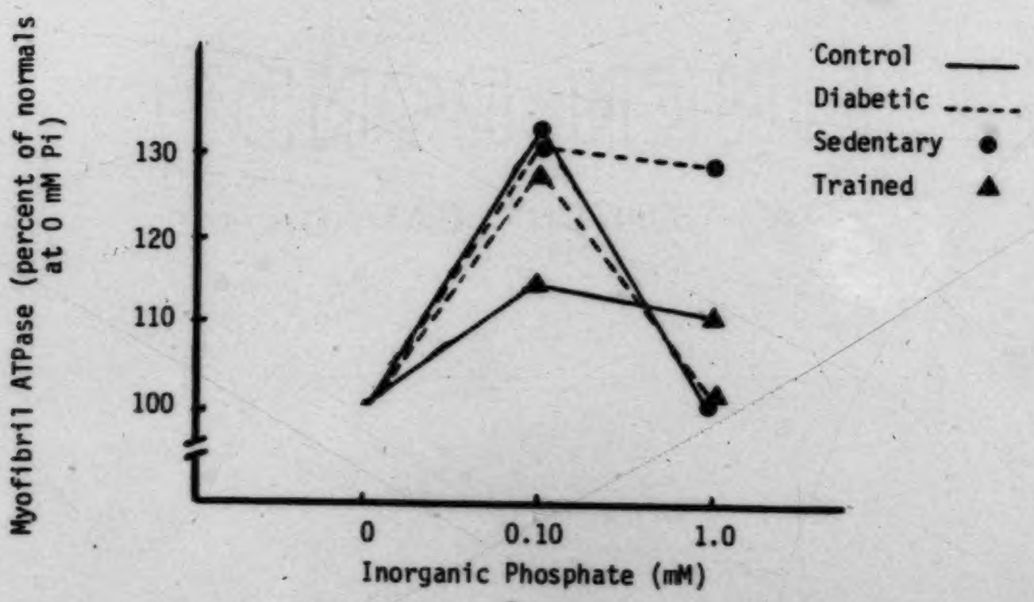

Figure 3b. Normalized Ca^{2+}-activated myofibril ATPase activities in response to varying Pi (pH 7.0)

were markedly greater than those of SD ($p < 0.001$). In the acidotic state (pH 6.5), a similar biphasic response to Pi was noted in SC ($p < 0.05$) which became diminished in diabetes (Figure 3a; Table 6). The Ca^{2+}-activated myofibril ATPase activity of SC exceeded that of SD at 0 mM Pi ($p < 0.05$) and at 0.10 mM Pi ($p < 0.01$) but not at 1.0 mM Pi.

A strong biphasic pattern characterized the response of the SC basal, Mg^{2+}-stimulated ATPase with increasing Pi at normal pH where activity increased to 250% ($p < 0.001$) then declined to 80% ($p < 0.001$) relative to normal (Figures 4a and 4b). In SD, a weaker biphasic response was observed, the activity being depressed relative to SC at 0.10 mM Pi ($p < 0.001$) and less so at 1.0 mM Pi. Reduced pH resulted in a continual increase in the SC basal ATPase reaching 123% of normal at 1.0 mM Pi ($p < 0.01$) (Figure 4a; Table 6). In SD, a biphasic pattern showed 111% activation at 0.10 mM Pi relative to normal ($p < 0.05$). SC and SD differed at pH 6.5 only at the highest Pi level where SC ATPase activity was much greater ($p < 0.001$).

Endurance swimming resulted in 15.5% and 80% increases in the Ca^{2+}-activated and basal myofibril ATPase activities respectively of trained hearts ($p = 0.05$) (Figure 5a). With increasing Pi concentrations, TC demonstrated an activation of the Ca^{2+}-activated ATPase at 0.10 mM Pi ($p < 0.05$) but only a minimal response at 1.0 mM Pi (Figure 5a; Figure 3b) in contrast to the biphasic response to SC (Figure 3a). At 0.10 mM Pi TC ATPase activity was enhanced 15.5% above normal ($p < 0.05$) followed by a nonsignificant decrease to 11.3% above normal at 1.0 mM Pi (Figure 3b). TC remained elevated relative to SC at the last Pi point ($p < 0.001$). No activation of the basal ATPase at pH 7.0 by moderate Pi (0.10 mM) occurred in TC as opposed to SC ($p < 0.05$) (Figures 4b and 5a). In TC, ATPase activity declined in 45.4% of normal at the higher Pi level ($p < 0.05$)

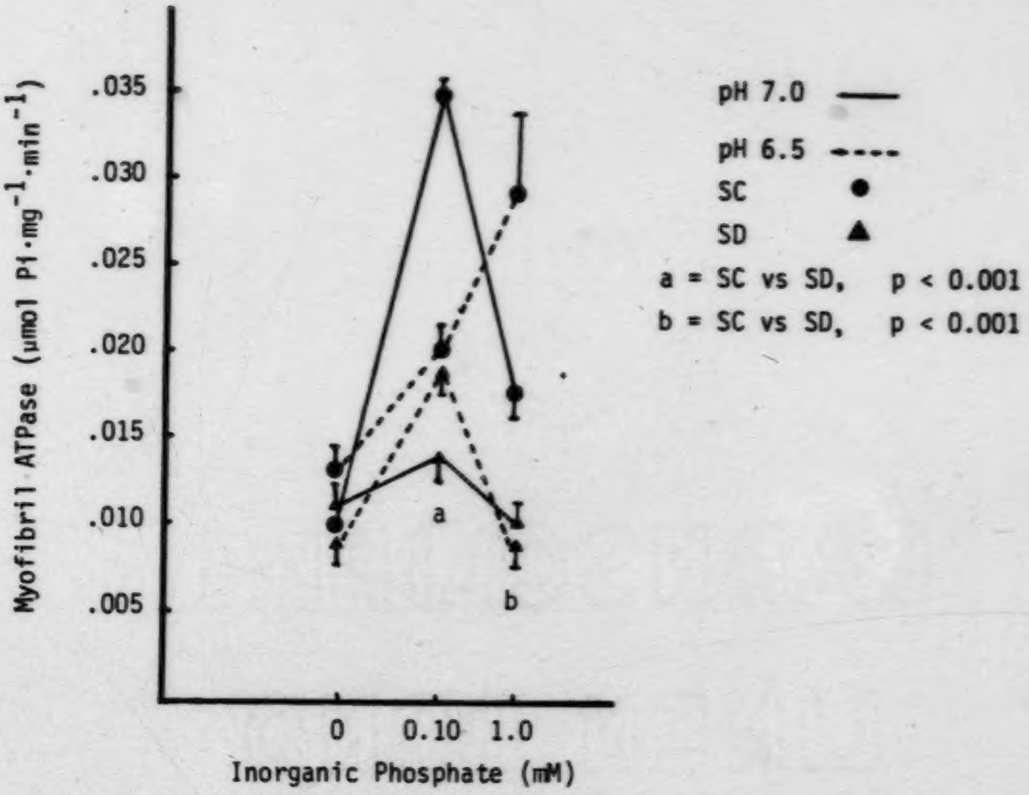

Figure 4a. Response of basal, Mg^{2+}-stimulated myofibril ATPase activity to increasing Pi in sedentary hearts

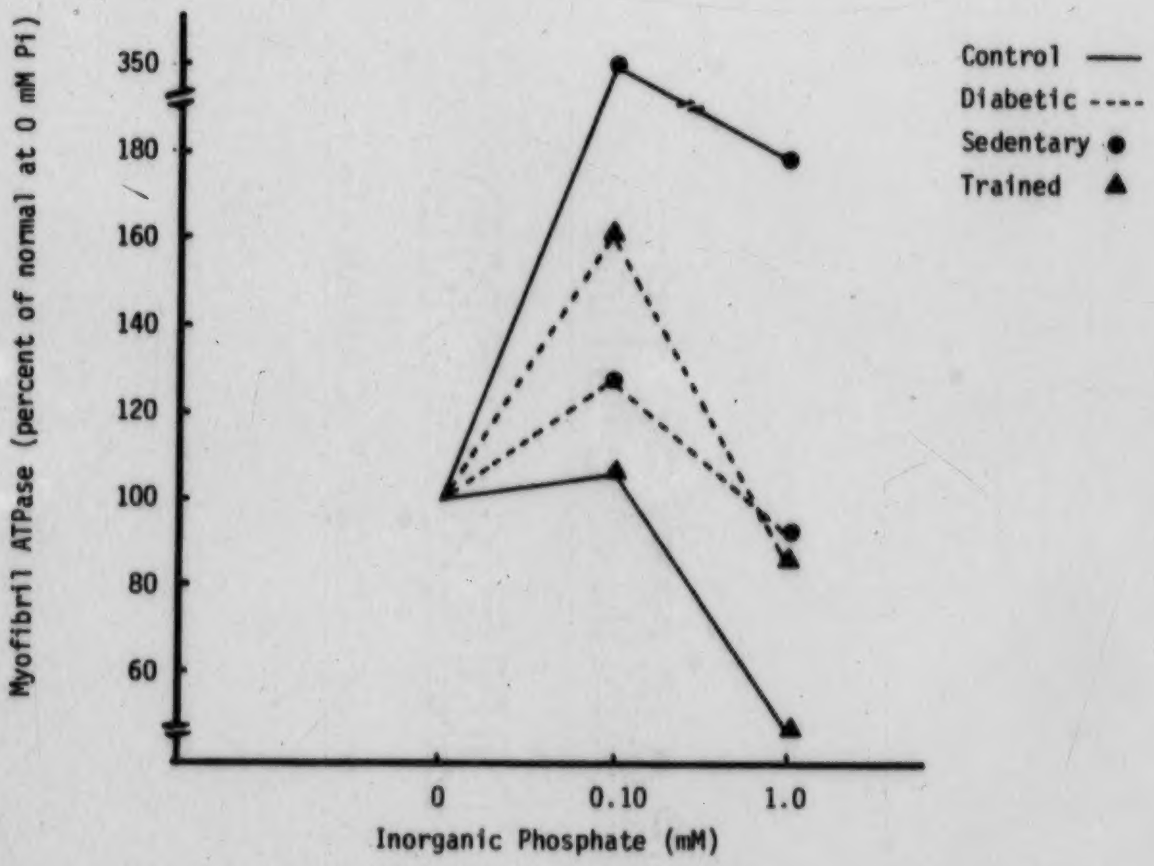

Figure 4b. Normalized basal myofibril ATPase activities in response to increasing Pi (pH 7.0)

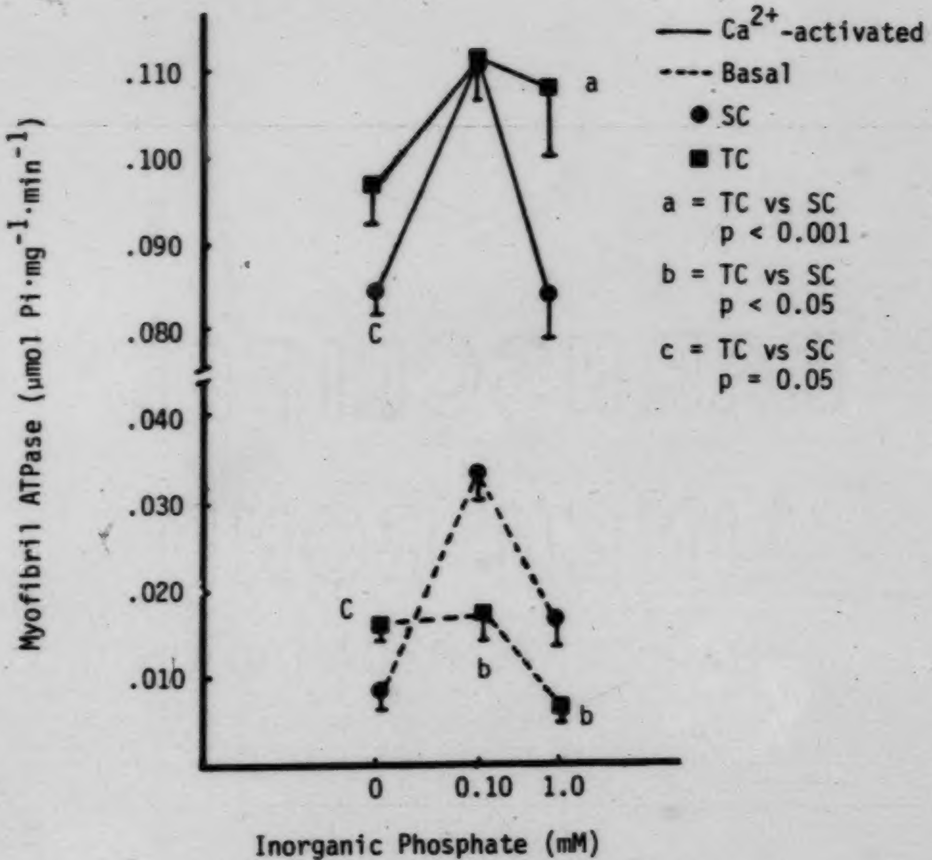

Figure 5a. Control sedentary and trained cardiac myofibril ATPase activities with increasing Pi (7.0)

which was depressed relative to SC ($p < 0.05$) (Figure 4b).

At pH 6.5, TC demonstrated a pronounced biphasic response to Pi, unlike pH 7.0, in the Ca^{2+}-activated state (115.6% activation above normal, $p < 0.001$; decline to 46.8% above normal, $p < 0.01$) (Figure 5b; Table 6). TC was elevated relative to SC at both 0.10 and 1.0 mM Pi ($p < 0.01$ and $p < 0.05$ respectively). The basal Mg^{2+}-stimulated ATPases of both TC and SC were enhanced by moderate Pi (38.8% and 53.8% respectively) while at the high Pi, TC decreased and was depressed relative to SC ($p < 0.01$) (Figure 5b).

Endurance training apparently had little effect on the normal Ca^{2+}-activated ATPase activity of the diabetic group at pH 7.0 (0.042 [SD] to 0.048 [TD] $\mu mol\ Pi \cdot mg^{-1} \cdot min^{-1}$) compared to the 15.5% increase in TC activity over SC ($p < 0.05$) (Figure 6). Both SD and TD were severely depressed relative to SC and TC ($p < 0.001$). No significant changes in basal ATPase accompanied swimming in either TC or TD although TC ATPase tended to increase slightly while that of TD decreased slightly (Figure 6). With intracellular acidosis (pH 6.5) no significant changes in either Ca^{2+}-activated or basal ATPase activities occurred in response to endurance training both in control and diabetic animals.

In contrast to the decreased sensitivity of the TC Ca^{2+}-activated ATPase activity to Pi, the TD group exhibited a definite biphasic response with a 29.1% activation relative to normal followed by a return to normal at 1.0 mM Pi (pH 7.0) (Figures 3b and 7). All TD values were greatly depressed relative to TC ($p < 0.001$). The basal ATPase activity of TD showed reduced sensitivity in both activation and inhibition by Pi where only 9% inhibition was observed as compared to 54.6% in TC (Figures 4b and 7). Reduction in pH resulted in a slight biphasic response of both the Ca^{2+}-activated and basal ATPases in TD (Table 6).

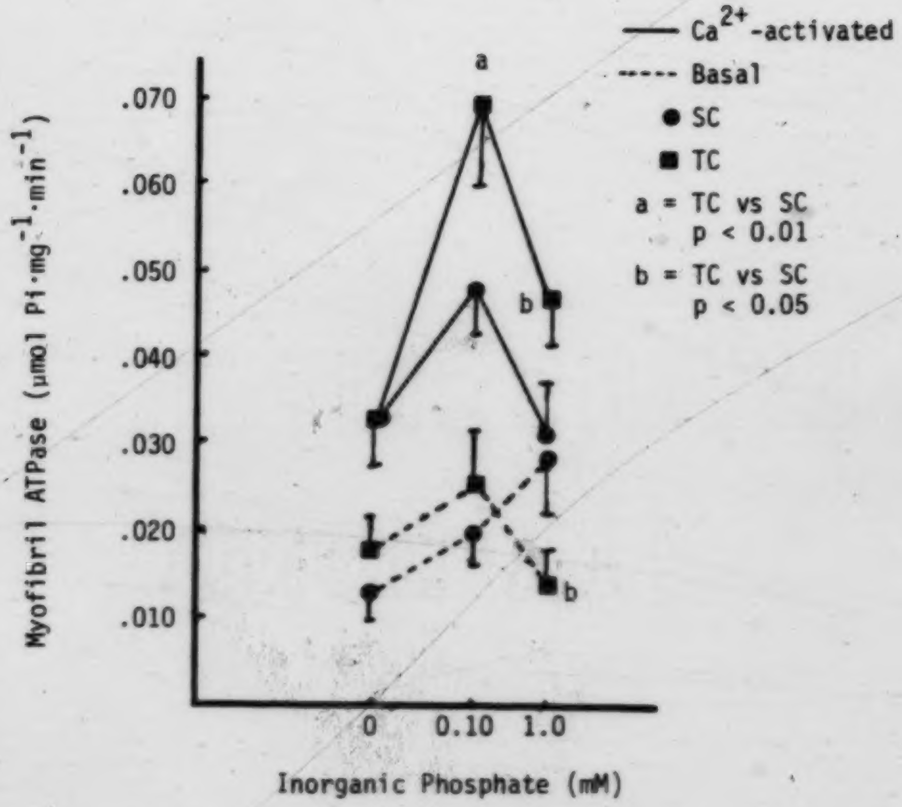

Figure 5b. Control sedentary and trained cardiac myofibril ATPase activities with increasing Pi (6.5)

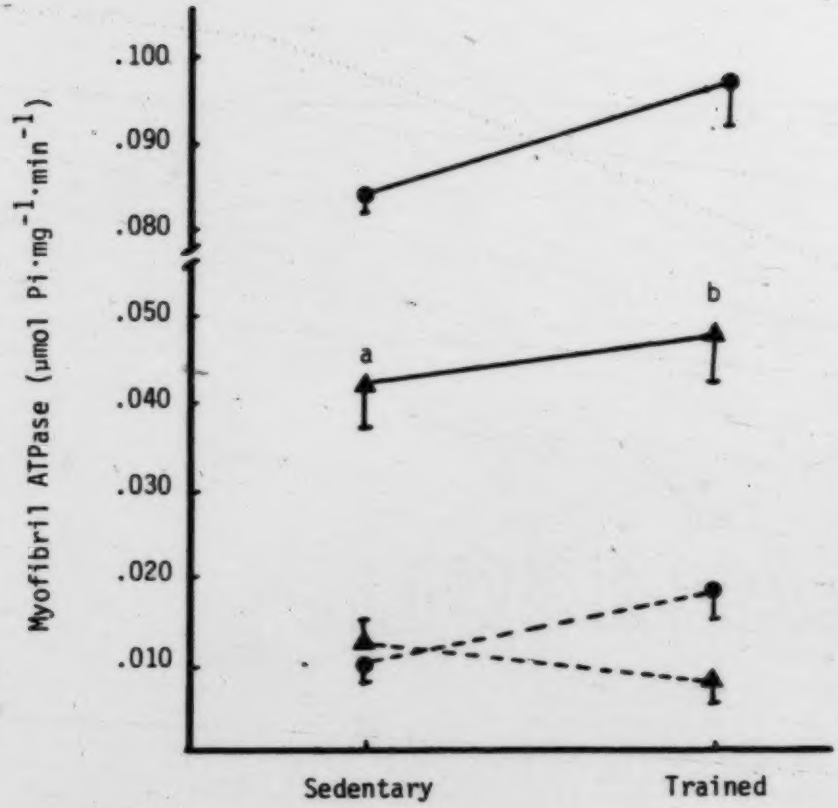

Figure 6. Myofibril ATPase response to endurance training in control and diabetic hearts (pH 7.0)

— Ca^{2+}-activated

---- Basal

● Control

▲ Diabetic

a = SC vs SD, $p < 0.001$

b = TC vs TD, $p < 0.001$

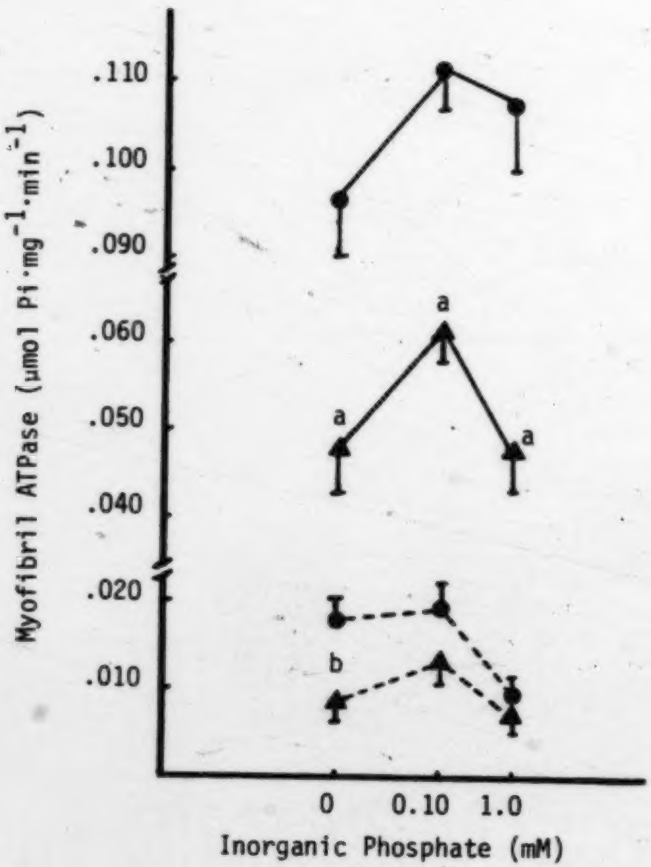

Figure 7.. Myofibril ATPase response to increasing Pi in trained control and diabetic hearts at pH 7.0

— Ca^{2+}-activated
---- Basal
● TC
▲ TD
a = TC vs TD, $p < 0.001$
b = TC vs TD, $p < 0.05$

Relative to its SD counterpart, the TD Ca^{2+}-activated ATPase showed a greater biphasic response to Pi with a similar extent of activation but much greater inhibition over the Pi range and at normal pH (Figure 3b). At pH 6.5, the SD and TD were similar. The accentuated biphasic response of TD relative to SD with increasing Pi was also observed in the basal, Mg^{2+}-stimulated ATPase (62.5% versus 27.5% activation respectively over normal (Figure 4b). Acidosis elicited a reverse of this pattern with SD becoming more biphasic relative to TD (111% versus 45% activation and 111% versus 36% inhibition respectively over normal) (Table 6).

TABLE 6

NORMALIZED* MYOFIBRIL ATPASE DATA AT pH 6.5

	Phosphate conc. (mM)	CONTROL		DIABETIC	
		Sedentary	Trained	Sedentary	Trained
Ca^{2+}-activated	0	100	100	100	100
	0.10	150	215.6	141.2	138.9
	1.0	96.9	146.8	117.6	119.1
Basal	0	100	100	100	100
	0.10	153.8	138.8	+211.1	145.5
	1.0	223.1	77.7	100	109.1

* values expressed as percent difference from normal or 100% (0 mM Pi)

CHAPTER IV

DISCUSSION

Anthropometric measurements of heart weight (HW), body weight (BW) and ratio of HW to BW (HW:BW) were comparable to those observed in previous investigations using a similar model of diabetes (Pierce and Dhalla, 1981; Penparkgul et al., 1980; Malhotra et al., 1981). The increased HW:BW ratio was attributable to the greater relative decline in BW as compared to that of HW. Decreased BW and HW in diabetics are probably related to the increased rates of protein degradation which accompany the insulin-deficient state (Rannels et al., 1970; Neely and Morgan, 1981) as well as to the depletion of adipose tissue (Neely and Morgan, 1981). With regard to endurance swimming, the HW changes of male rats were negligible while BW decreased by 10.5% compared to sedentary controls. The absence of HW changes are consistent with previous reports while the reduced weight gain occurred to a lesser extent than in other swimming studies (Penparkgul et al., 1978; Guisti et al., 1978; Malhotra et al., 1976). This difference may be related to the intensity and duration of the training programs. In the present investigation tail weights and shorter swims were employed as compared to other programs which employ longer, unweighted swims. In this respect, the weighted, 30-minute bouts used by Wilkerson and Evonuk (1971) failed to elicit BW changes over a 10-week period. The increased HW:BW ratio incurred with training can be attributed to the modest decline of BW (Penparkgul et al., 1978). This lesser gain of BW over the training period as compared to sedentary animals and the minimal change in HW are considered to be normal responses to swim training (Bhan and Scheuer, 1972). Retardation of weight gain during endurance training of male rats has been related to decreased food consumption (Nance et al., 1977). The lack of increase in cardiac mass indicates that training adaptations did not include cardiac hypertrophy.

Plasma glucose values employed as a parameter of the diabetic state indicated that moderate diabetes (680 ± 33.5 $mg \cdot dl^{-1}$) resulted from streptozotocin injection within 7 days and remained stable during the experimental period. This constitutes a normal extent and pattern of response in male Wistar rats with this dosage range (600-650 $mg \cdot dl^{-1}$) (Malhotra et al., 1981; Penparkgul et al., 1981). At 8-weeks post-injection values from this study were in the range of 400-900 $mg \cdot dl^{-1}$. Studies employing male Sprague-Dawley rats and a 60-65 $mg \cdot kg^{-1}$ dosage of streptozotocin have obtained similar response patterns but at a slightly lower range of plasma glucose values (400-600 $mg \cdot dl^{-1}$) (Ganda et al., 1976; Heyliger et al., 1982; Pierce and Dhalla, 1981). Thus, species differences may account for some of the discrepancies between the plasma glucose values of this study and those of others. A high degree of variability was also observed in the plasma glucose values of the diabetic groups which may also have contributed to the higher mean values in this study. The reasons for this variability are not evident. Higher plasma glucose values may also have been related to the choice of the penile dorsal vein for injection of streptozotocin possibly permitting better delivery of the drug and allowing immediate knowledge of the adequacy of the injection.

The endurance training program resulted in a 15.5% elevation of the maximal, Ca^{2+}-activated myofibril ATPase activity ($p = 0.05$) and in an 80% increase of the basal, Mg^{2+}-stimulated ATPase activity ($p = 0.05$) over that of controls. These adaptations are similar but of a lesser magnitude than studies reporting significant increases of myosin and actomyosin ATPase activities (Hearne and Gollnick, 1961; Wilkerson and Evonuk, 1971; Bhan and Scheuer, 1972 and 1975; Malhotra et al., 1976; Giusti et al., 1978; Penparkgul et al., 1980). Furthermore, Rupp (1981) reported a 10% increase in the Ca^{2+}-activated myofibril ATPase activity of endurance-swum hearts. It is possible that the extent of the ATPase change is associated with duration of the swimming program since studies employing 150 minute versus 90 minute daily bouts tend to exhibit greater ATPase activities (Bhan and Scheuer, 1972; Malhotra et al., 1978). The type of in vitro preparation is probably important as well, since myofibril ATPase results from this study are in line with those of Rupp (1981). The possibility that the ATPase enhancement may be part of a neurogenic response to repeated water immersion itself has been discounted by Penparkgul et al. (1980), who showed that animals immersed and supported in water retained the ATPase characteristics of sedentary animals. Whether forced swimming may have a neurogenic component has not been determined.

The biochemical mechanisms responsible for the altered myofibril ATPase activity with swimming are not yet clear. A shift to almost complete dominance of the myosin V_1 isoenzyme which exhibits a greater ATPase activity relative to the V_2 and V_3 forms has been reported (Rupp, 1981). Rupp (1981) states that swim-training alters myofibrillar ATPase activity to a lesser degree than the actin-activated myosin ATPase of other swimming studies (Bhan and Scheuer, 1975; Medugorac, 1975) and further suggests that the myofibrillar ATPase best represents the changes in cross-bridge cycling rate and velocity of shortening in a quantitative manner. Therefore, the enhanced myofibril ATPase obtained in the present investigation could be explained by a similar shift in myosin isoenzyme synthesis and manifest as the higher enzymatic activity.

Additional mechanisms which have been identified include further modifications to myosin in the region of the active site and on the light chains. Medugorac (1975) reported a greater amount of the LC_1 light chain with swimming although the significance of this adaptation is not understood. Running-trained hearts which displayed elevated myosin Ca^{2+}-ATPase activities demonstrated a greater extent of P-light chain phosphorylation. The significance of this finding was suggested to arise from the augmented contractility due to potentiation of the myosin ATPase and cross-bridge activation by the phosphorylated light chains (Resink et al., 1981[b]). Since no precautions were taken in this study to eliminate kinases and phosphatases from the in vitro preparations, the contribution of this mechanism to the observed elevation in ATPase activity may be of some importance. Studies employing various chemical agents and probes that interact with the catalytic region of myosin (KCl, ethylene glycol and SH_1 and SH_2 modifying agents) have revealed conformation changes and altered thiol group reactivities in swim-trained hearts (Bhan and Scheuer, 1975; Bhan et al., 1975). With regard to thin filament adaptations, Resink et al. (1981[a]) demonstrated that TNI phosphorylation was not altered with endurance running. Other thin filament parameters have not been examined with chronic exercise.

In consideration of the possible mechanism(s) responsible for the enhanced ATPase activity of trained hearts, results from this investigation suggest that the regulatory influence of inorganic phosphate ions (PO_4^{3-}) on the myofibril ATPase activity may become modified. Control (non-diabetic) hearts demonstrated an attenuation of the Ca^{2+}-activated ATPase response to increasing phosphate (Pi) levels with endurance swimming. This was observed as a flattening of the distinct, biphasic response pattern elicited by the ATPase of sedentary hearts where slightly less activation by 0.10 mM Pi and significantly less inhibition by 1.0 mM Pi ($p < 0.05$) could be seen. In addition, a plateau or ceiling effect in the activation by moderate Pi was observed in both sedentary and trained hearts. The basal-Mg^{2+} stimulated ATPase revealed a similar biphasic response to Pi. Inorganic phosphate ions have been shown to reduce tension and immediate stiffness in chemically-skinned myocardial strips (Herzig and Ruegg, 1977; Herzig et al., 1981) as well as reducing tension generated by whole isolated perfused hearts (Solaro et al., 1980 [a, b]). In addition with millimolar Pi myofibril ATPase activities and actin-cofactor activity are depressed over a range of activating Ca^{2+} levels (Solaro et al., 1980[a, b]) but at a given level of tension in myocardial strips, ATPase rates are elevated (Herzig et al., 1981). This elevated ATPase activity has been interpreted as an increased cross-bridge cycling rate where tension maintenance would occur at a much greater energy cost (Herzig et al., 1981). These observations may be related to the present data in the following scheme: Low, micromolar levels of Pi may enhance ATPase activity as suggested by Herzig et al. (1981) and observed in this study. The site and mechanism for this activation is not known but since the effect is Ca^{2+}-independent, the site of Pi's influence is probably located on myosin. Solaro et al. (1980[a]) postulated that the oxidation state of vanadate, and therefore possibly of Pi, could determine its intracellular action. Therefore the alteration in cross-bridge kinetics by Pi may be related to its high negative charge. As a result of the increased ATP hydrolysis, Pi accumulation in the region of the active site may ensue and begin to exert a negative feedback control on product release by the ATPase. This would explain the observed inhibition in this study at the higher Pi concentration. The possibility of product inhibition within the actomyosin ATPase cycle has been previously suggested (Chock et al., 1981; Goodno, 1979). Product inhibition may indirectly be the result of an inability of the ATP-CPK (creatine phosphokinase) system (Bessman et al., 1980) to effectively remove Pi from the active site region and rephosphorylate ADP under conditions where Pi levels may become relatively high. The observed decrease in sensitivity to Pi of the ATPase with training may be beneficial in that it would permit maintenance of the high rates of ATP hydrolysis in the face of possible Pi accumulation rather than the depression observed by Solaro et al. (1980[a, b]). Since tension development may become enhanced with training (Schaible and Scheuer, 1979), it appears that reduced sensitivity to Pi could also allow maintenance of the force-generating capabilities of cardiac muscle as ATP rates become elevated. At basal levels of ATPase activity, the lack of activation but greater inhibition by Pi in trained hearts could perhaps maintain low ATP use and low resting tension under conditions where Pi might become elevated. Inotropic stimulation has been cited as a cause of reduction of the CP:C ratio (Herzig and Ruegg, 1977) and would further enhance Pi accumulation in the inotropic response to exercise. Thus, the ability of the cardiac myofibrils to keep ATP hydrolysis low during the relaxation phase of the cycle in spite of increased Pi could possibly contribute to a "conservation" of ATP during diastole.

Streptozotocin-diabetes led to a profound reduction in the cardiac Ca^{2+}-activated myofibril ATPase activity ($p < 0.001$) with little change in the basal, Mg^{2+}-stimulated condition. The depression in Ca^{2+}-activated myofibril ATPase activity corresponds with that reported by Pierce and Dhalla (1981) while myosin and actomyosin preparations yield similar results (Dillman, 1980; Malhotra et al., 1981). A number of cellular mechanisms could be contributing to the altered enzymatic activity. Both

Dillman (1980) and Malhotra et al. (1981) observed a marked change in the predominance of V_1 and V_3 myosin isoenzymes where the V_3 form with its 10-fold lower ATPase rate become more abundant. Structural alterations at or near the myosin active site and modification of thiol group function were recorded and implicated in the depressed ATPase rates (Pierce and Dhalla, 1981). The dependence of myofibril ATPase on a range of physiological free Ca^{2+} concentrations appear not to be influenced in the diabetic state although absolute ATPase activity is depressed over the entire Ca^{2+} range (Pierce and Dhalla, 1981). As regards the regulatory role of phosphate ions (Pi) on myofibril ATPase activity of diabetic hearts, the present work revealed that both the basal Mg^{2+}-stimulated and the Ca^{2+}-activated ATPase response of sedentary hearts became modified in diabetes with loss of the sharp biphasic pattern to increasing Pi. In particular, the Ca^{2+}-activated ATPase showed no inhibition of the ATPase by millimolar Pi. The impact of the depression of absolute ATPase activity combined with the loss of feedback regulation on the ATPase by Pi accumulation could result in an uncoupling of ATP hydrolysis and force-generation by the myofibrils. The loss of Pi regulation would serve to magnify the reported loss of force-generating capabilities associated with the reduced ATPase (Fein et al., 1980).

The contribution of altered protein metabolism and thyroid hormone levels to the reduced ATPase activity in diabetes is also of some importance. Insulin is an important regulator of protein synthetic and degradative pathways (Neely and Morgan, 1981). Administration of insulin reversed the myosin isoenzyme expression to that of a non-diabetic state (Dillman, 1980). The reduced amount of insulin in the streptozotocin-diabetic rats plus the probable elevation of ketones resulting from incomplete lipid β-oxidation in uncontrolled diabetes would inhibit protein synthesis and permit greater activity in the degradative pathways (Pain and Garlick, 1974; Neely and Morgan, 1981). Increased excretion rates of the myofibril-specific amino acid 3-methyl-N-histidine have been demonstrated and indicate that myofibril degradation is enhanced in the diabetic state (Marchesini et al., 1982). Ultrastructural studies have revealed myofilament disarray and myofibrillolysis providing additional evidence for the disruption of myofibril structural integrity (Giacomelli and Winer, 1979) perhaps leading to loss of functional integrity. Endogenous proteases which may become more active in diabetes have been implicated in this degradation (de Martino, 1982; Dahlmann and Reinauer, 1981).

With regard to thyroid hormone, significant reductions in circulating levels of T_3 and T_4 have been observed with diabetes (Dillman, 1980; Fein et al., 1980). Under normal conditions thyroid hormones exert an anabolic effect while reduced levels have been shown to alter the myosin isoenzyme pattern to the slower V_3 dominance (Yazaki and Reuben, 1975; Hoh et al., 1979). Pharmacological but not physiological doses of T_3 and T_4 hormones administered to diabetic rats successfully normalized the myosin isoenzyme pattern and ATPase activity (Dillman, 1981). Whether this reflects reduced thyroid-hormone responsiveness in diabetes or a diabetes-related cellular disturbance sensitive to thyroid hormone has not yet been determined. The contribution of some thyroid hormone-related pathophysiology leading to altered myofibril ATPase activity in diabetes cannot be dismissed.

Finally, the possibility of a direct effect of streptozotocin on the cardiac ATPase activity should be addressed. Administration of the glucose analog, 3-O-methyl glucose which blocks the effect of streptozocin at the pancreatic membrane and prevents the drug-induced diabetes also prevented alterations in actomyosin ATPase activity. This substantiated the lack of a direct toxic effect of the drug on the myocardium (Malhotra et al., 1981).

Endurance training failed to reverse the depressive effects of diabetes of the absolute Ca^{2+}-activated myofibril ATPase activity. However, training did restore normal responsiveness of the ATPase to varying Pi concentrations resuming the biphasic pattern seen in sedentary control hearts. The mechanism by which this occurs is unknown but indicates that ionic regulation of ATPase in trained diabetic hearts is somehow improved and could serve to maximize the already low activity. Normalization of the Pi influence suggests that an adaptation of the myosin head influencing cross-bridge kinetics has occurred. Endurance training has also been shown to reduce plasma insulin levels while increasing insulin-receptor number and/or affinity as well as increasing the anabolic effects of insulin (Terjung et al., 1979; Iancrede et al., 1982; Reaven and Chang, 1981). Thus, the restoration of some normal regulatory mechanisms of ATPase with training could be the result of improved sensitivity of protein synthetic degradative pathways to the low insulin levels present in diabetes. Myofibril degradation may become reduced while protein synthesis could perhaps shift the myosin isoenzyme expression to a more normal pattern. Whether the various isoenzymes exhibit different responses to regulatory ions has not been examined. The greater dependence of diabetic hearts on the oxidation of fatty acids for ATP production leads to accumulation of ketones. As well, conditions which increase heart work such as hypoxia, ischemia and/or elevated preloads and afterloads also lead to a large accumulation of lipid substrates and impairment of oxidative metabolism (Miller, 1979; Opie et al., 1979; Feuvray et al., 1979). Endurance training may lead to improved utilization of lipid substrates and ketones by myocardial tissue which would reduce the inhibitory effect that ketones exert on protein synthesis (Neely and Morgan, 1981). This could further influence myofibril protein preservation and function in diabetes.

In the absence of exogenous Pi, acidosis elicited a uniform depression of Ca^{2+}-activated ATPase activities of all groups with little effect on basal ATPase levels. Thus, intracellular acidosis may not be important in itself as regards myofibril ATPase activity of trained or diabetic hearts. However, reduced pH altered the response to increasing Pi levels. Sensitivity to Pi was greatly exaggerated in trained control hearts at pH 6.5 maintaining the biphasic pattern of response. Relative responses of the diabetic hearts were similar at both pH. The apparent interaction of H^+ ions and Pi may occur in the region of the myosin active site where it has been postulated that H^+ ions participate in product release from the myosin ATPase (Shukla and Levy, 1979). Thus, pronounced intracellular acidosis appears to override the training-induced alterations to Pi regulation. Since intracellular pH has been reported as low as 6.4 with muscular fatigue (Gonzales et al., 19), this interaction of H^+ with other regulatory ions may represent a limiting mechanism in endurance exercise.

CHAPTER V

CONCLUSIONS, LIMITATIONS AND RECOMMENDATIONS

Conclusions

The present investigation:

1) confirmed the marked influences that endurance swimming and uncontrolled diabetes mellitus have on cardiac myofibril ATPase activity.
2) demonstrated that training results in a reduced sensitivity of myofibril Ca^{2+}-activated ATPase activity to inorganic phosphate ions. This may permit maintenance of higher cross-bridge cycling rates during periods of increased heart work.
3) demonstrated that endurance swimming cannot reverse the depressive effects of uncontrolled diabetes on cardiac myofibril ATPase activity.
4) indicated that endurance swimming may normalize some aspects of ionic regulation of the myofibril ATPase in diabetes. This was observed as a response of TD to increasing Pi which resembled that of SC.
5) indicated that the mechanisms which are responsible for the depressed myofibril ATPase activity with diabetes may not be the same as those directly contributing to the enhanced ATPase activity with training.

Therefore, endurance training alone does not appear to be an effective method for restoring normal cardiac function in the cardiomyopathy associated with diabetes.

Limitations and Recommendations

Factors which have limited the extent to which this research can be generalized or which require further work include the following:

1) The model utilized was not controlled by insulin administration. This has limited relevance with regard to controlled human diabetes since insulin injection is standard treatment in Type I diabetes. Therefore it is recommended that further studies include insulin-controlled groups in order to determine: a) whether the observed abnormalities in ATPase activity and regulation are directly and/or indirectly due to insufficient insulin at the cellular level or b) whether the observed dysfunctions occur as a result of diabetes but independent of insulin.
2) Only male Wistar rats have been studied. Whether similar results would be obtained with female animals cannot be assumed, therefore further work could evaluate this problem.
3) The question of the usefulness of animal models of human disease is itself a major limiting factor. Since no one animal model accurately mirrors the pathophysiology of human diabetes, which is itself diverse, application of results from animal research to human diabetes must be treated cautiously. However, for practical and ethical reasons, this problem cannot be eliminated at this point it time.
4) Criteria for diabetes should also include measurement and analysis of weekly body weight changes and of 24-hour urine volumes. In addition, animals should be uniformly fasted or non-fasted prior to urine and glucose sampling.
5) Chronic treatment with non-human insulin which occurs in human Type I diabetes may result in immunlogical responses. These responses are generally not distinguishable in the literature from the pathophysio-

logical effects of chronic diabetes itself. This may present problems when investigating the pathophysiology of human controlled diabetes.

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APPENDIX A

REVIEW OF LITERATURE

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REVIEW OF LITERATURE

I. MUSCLE STRUCTURE

Gross Morphology

The structure of striated muscle cells was first described for skeletal muscle by Hanson and Huxley (1953) and has since been found to be homologous in working myocardial cells (Sommer and Johnson, 1979). The myofiber is the gross morphological and functional unit of the striated muscle. Under the light microscope fixed and stained myocardial cells (excluding conductive and pacemaker cells) appear as numerous fibers each enclosed in a plasma membrane or sarcolemma. The myofibers are connected to one another end-to-end by tight junctions known as intercalated discs which appear as densely-staining transverse bands characteristically located at right angles to the long axis of the cardiac myofibrils. These discs represent true cell-to-cell junctions which serve as low resistance pathways facilitating cell-to-cell electrical flow and are without true counterparts in skeletal muscle (Adams and Schwartz, 1980; Katz, 1977). The myofibers bifurcate and connect with adjacent fibers to form an intricate three-dimensional network in which cell sizes may vary between 50 and 100 mm in length and 10 to 20 μm in diameter. Skeletal myofibers, in contrast, appear as uniform, long parallel fibers grouped into fascicles with cellular dimensions ranging from 10 to 100 μm in diameter and with variable lengths (Adams and Schwartz, 1980).

Within the cell, bordered by the sarcolemma, cellular components are dominated by the myofibrils which occupy 50 to 60% of the cell volume

(Hirakow, 1980) and appear as bundles of thin longitudinal elements having a characteristic repeating pattern of light and dark transverse bands giving the fiber a striated appearance. Other structural features of the cardiac myofiber include mitochondria, much more abundant than in skeletal muscle cells, a centrally-located nucleus as opposed to numerous peripherally located nuclei in skeletal muscle cells and an extensive internal membrane system, the sacroplasmic reticulum, with tubular invaginations of the sarcolemma, the transverse tubule system (Adams and Schwartz, 1980).

Sarcolemma

Both skeletal and cardiac sarcolemma reveal three distinct layers upon electron microscope observations. The basic cell unit membrane is referred to as the plasmalemma exhibiting a bilayered structure of 7 to 9 nm in width (Langer, 1978). External to the unit membrane lies a 50 nm-thick layer termed the glycocalyx and consists of two structural components: the surface coat (20 nm thick) and the external lamina (30 nm thick) (Bennett, 1963). In the myocardium the glycocalyx follows the unit membrane as it invaginates the cell to form the transverse tubules (T-tubules) whereas in skeletal muscle the glycocalyx does not extend into the T-tubule system (Langer, 1978). Histochemical studies have demonstrated the polyanionic nature of the glycocalyx ascribable primarily to the abundance of acidic mucopolysaccharides, glycoproteins and sialic acid residues in the coats. This contributes to the negatively charged polar head groups of the unit membrane phospholipids and creates an extracellular region with a high capacity for cation binding. The negatively-charged carboxyl groups of sialic acid residues present next to the lipid bilayer and on the external lamena have particular significance in regulating membrane cation permeability (Langer, 1978; Adams and Schwartz, 1980). The heart muscle has 54 times more sialic acid relative to its external volume as compared to skeletal muscle which translates to 400% greater surface cation-binding capacity per unit volume per cell in cardiac muscle (Langer, 1978).

Transverse Tubule System

The sarcolemma of striated muscle is vesiculated and indented at regular intervals. The indentations represent sites of tubular membrane invaginations of the plasmalemma into the cell interior and are known as the transverse tubules (T-tubules). The T-tubules of cardiac muscle invaginate primarily at the site of the Z-line of the sarcomere penetrating to the center of the myofiber and occasionally bifurcating to extend longitudinally between adjacent myofibers (Sommer and Johnson, 1979). Skeletal muscle T-tubules invaginate the cell at the site of the H-I band of the sarcomere and also bifurcate horizontally. Three major morphological differences have been described for cardiac and skeletal T-tubules: 1) the lumen of the cardiac T-tubules is variable but generally greater than that of skeletal T-tubules, 2) cardiac T-tubules are heavily vesiculated containing numerous membrane-bound particles and includes the glycocalyx unlike skeletal muscle, and 3) skeletal T-tubules have consistent orientation to the intracellular sarcoplasmic reticulum (SR) called triads whereas those of cardiac muscle are more randomly coupled to the SR (Adams and Schwartz, 1980).

Sarcoplasmic Reticulum

The sarcoplasmic reticulum (SR) represents a tissue-specific differentiation of the endoplasmic reticulum. It constitutes an intracellular membrane system which is discontinuous with the plasmalemma but continues with internal membranes such as the nuclear envelope (Adams and Schwartz, 1980). A network of tubular elements surrounds each myofibril, most of which being smooth reticulum with small amounts of ribosomal reticulum. The SR functions as a prime regulator of myoplasmic Ca^{2+} by way of Ca^{2+} storage, Ca^{2+} release and as the site of excitation-contraction coupling (Tada et al., 1978). Several structural differences have been identified between skeletal and cardiac SR. Skeletal SR is arranged in parallel fashion and anastomose freely in the region of the A-bands whereas cardiac SR displays a more random orientation to the myofibrils. In addition, skeletal sarcotubules are confluent in the region of the A-I band junction and comprise the enlarged channels known as terminal cisternae. Pairs of parallel terminals run transversely across the myofibrils in close association with the T-tubules to form a triad. These structures appear to be linked by junctional processes which are extensions of the SR membrane. In cardiac muscle, the SR terminates in sarcotubular dilations which closely appose the T-tubule and sarcolemma via junctional processes. The close association in cardiac SR of extra- and intra-cellular structures indicates a close functional relationship (Adams and Schwartz, 1980; Van Winkle and Entman, 1979). In total skeletal muscle contains a much greater amount of SR per cell or myofibrillar volume than does cardiac muscle (Van Winkle and Entman, 1979).

The SR associated with the plasmalemma or T-tubule is known as junctional SR (JSR) while non-junctional SR is termed free SR (FSR). Different protein compositions of these regions may reflect functional differences. Isolated SR assumes the form of closed spheres and is composed of seven proteins plus a lipid component; five proteins are considered intrinsic to the membrane plus the two loosely-bound extrinsic proteins (Van Winkle and Entman, 1979; Tada et al., 1978). Major intrinsic proteins include the 100,000 dalton Ca^{2+}-ATPase and a proteolipid. The Ca^{2+}-ATPase acts as a Ca^{2+} pump having a high affinity for Ca^{2+} and allows for rapid rates of Ca^{2+} and binding across the SR during relaxation. Whether this protein spans the SR membrane or traverses only part of it remains unsettled (Adams and Schwartz, 1980; Tada et al., 1978). The role of proteolipid is unclear but appears to be involved in Ca^{2+} transport. Phospholamban, a 22,000-dalton protein acts to enhance Ca^{2+}-ATPase activity and Ca^{2+} uptake when phosphorylated (Tada et al., 1978). The extrinsic proteins calsequestrin and high-affinity Ca^{2+}-binding protein act as Ca^{2+} sinks within the SR and/or may play a role in Ca^{2+} translocation (Adams and Schwartz, 1980).

MYOFIBRILS

Morphology

Cardiac and skeletal myofibrils are the same morphologically and very similar functionally (Adams and Schwartz, 1980). The sarcomere is the fundamental morphological unit of the striated muscle and is delineated by two successive, narrow dark lines known as Z-lines. At rest, this distance in cardiac muscle is between 2 and 3 μm (2.2 μm is optional) (Katz, 1977). Within the sarcomere is a darkly-staining, centrally-located region known as the A-band. When viewed through cross polaroids this area rotates polarized light in a birefringent manner and is thus anisotropic (A). This birefringence indicates the presence of a highly-ordered, parallel array of macromolecules (Katz, 1977). At either end of the A-band are more lightly staining sections known as I (isotropic)-bands which are bisected by the dark Z-lines. At rest, the central region of the A-band appears less dense and is termed the H-band. It is bisected by a dense M-line. In summary, a sarcomere lies within two Z-lines and contains a single A-band and two adjacent half I-bands (Katz, 1977; Adams and Schwartz, 1980).

Ultrastructure

Electron microscope and X-ray diffraction studies have revealed the ultrastructure of the myofibrils (Huxley and Hanson, 1953). The cross-striated appearance of muscle was shown to arise from the arrangement of the contractile proteins into overlapping thick and thin filaments. The thicker myosin filaments, with dimensions of 150 Å in diameter and 1.5 to 1.6 μm (microns) in length (McCubbin and Kay, 1980) constitute a parallel arrangement that extends throughout the length of the A-band and is maintained by centrally-located connections at the M-line. In the I-band, the thinner actin filaments, 60 Å in diameter, attach to and extend 1 μm from the Z-line to interdigitate with the myosin filaments at rest. It is this interdigitation which accounts for the denser region of the A-band and the extent depends on sarcomere length. The thin filaments also consist of tropomyosin and troponin associated with the actin. Under higher magnifications electron micrographs of glycerol-extracted muscles showed that the peripheral ends of the myosin filaments are covered with numerous projections which are capable of establishing links with the adjacent thin filaments, hence their designation as "cross-bridges" (Adams and Schwartz, 1980; Huxley and Brown, 1967).

In cross-section, the A-bands contain a hexagonal array of thick filaments, each of which is surrounded by six thin filaments which lie at trigonal points between the adjacent thick filaments. In the I-band, only thin filaments can be observed in a less orderly array. The M-band contains thick filaments held together in a hexagonal array of cross-links (Katz, 1977).

The less dense I-band consists only of actin and regulatory proteins, while the H-zone is composed only of myosin filaments. The widths of these regions are determined by the degree of actin and myosin overlap while the A-band width remains relatively constant. The H-zone is devoid of myosin cross-bridge projections. The opposite polarity of the thick and thin filaments in each half of the sarcomere dictates that the thin filaments penetrating the A-band may only interact with the cross-bridges of the same half and thus during contraction, the thin filaments are pulled towards the center of the sarcomere as proposed in the sliding filament theory (Katz, 1977; Huxley, 1969).

MYOSIN

Molecular Characteristics

Myosin has been established as the chief constituent of the thick filament of striated muscle (Katz, 1970). Its selective extraction from skeletal muscle led to the disappearance of the A-band thick filaments (Huxley and Hanson, 1953) while aggregates of isolated skeletal and cardiac myosin monomers were indistinguishable from their respective thick filaments (Katz, 1970). Upon isolation, the myosin molecule appears as a hexameric, elongated molecule possessing a longer rod-like filamentous "tail" with two globular projections or heads at the other end (Meullar, 1974; Adams and Schwartz, 1980). The filamentous tail imparts structural rigidity to the thick filament and exists as a coiled-coil of two α-helices which extend into the heads giving rise to the paired globular structures which possess the enzymatic and actin-building characteristics of myosin (Katz, 1977).

Extraction of cardiac myosin yields only 1/3 to 1/2 the amount of protein as skeletal myosin extracted from their respective muscles. Studies employing ammonium sulfate fractionation, light-scattering methods and measurements of sedimentation and diffusion coefficients and viscosity have provided a wide range of molecular weights, this variability being attributed to the large molecular size, tendency towards aggregation and dependence of some techniques on protein concentration (Katz, 1970). The accepted value now lies between 470,000 and 500,000 d (daltons) (Mannherz and Goody, 1976; Katz, 1970).

The hydrodynamic properties of intrinsic viscosity, diffusion and sedimentation coefficients are demonstrably similar in skeletal and cardiac myosin (Katz, 1970). The physicochemical properties of both myosins indicate a highly asymmetric structure with molecular dimensions of 1600-1700 Å in length, 24 Å in width and a diameter of 70 Å in the terminal golbular heads obtained from light-scattering methods (Katz, 1970; Morel and Pinset-Harstrom, 1975). Optical rotatory dispersion measures indicate that approximately 54 to 60% of the peptide chains exist in the α-helical configuration in both skeletal and cardiac myosins (Katz, 1970).

Proteolytic digestion of the myosin molecule has reveaed the existence of several subunits (Lowey et al., 1969; Katz, 1970). Controlled exposure to the proteolytic enzyme trypsin releases two high-molecular weight components having different sedimentation velocities in the analytical centrifuge. The more rapidly sedimenting fragment, termed heavy meromyosin (HMM), retains the enzymatic ATPase and actin-binding capabilities of the myosin molecule, but unlike the entire molecule is soluble in solutions of low ionic strength (< 0.3 M). A molecular weight of approximately 350,000 d has been reported for skeletal myosin (Katz, 1970) while Muellar et al. (1969) measured cardiac HMM at 345,000 d. The lighter component of tryptic digestion, light meromyosin (LMM), possesses neither the enzymatic or actin-binding characteristics of HMM, but has solubility properties similar to native myosin where aggregation is observed in low ionic strength solutions (Katz, 1970, Star and Offer, 1973). Skeletal LMM molecular weight is given as 150,000 d (Katz, 1970) while that of cardiac as 145,000 d (Mueller et al., 1969). The maximum length of the LMM portion is about 900 Å and contains 1266 amino acid residues per mole of 150,000 MW. The two α-helix peptide chains form a parallel coiled-coil structure beginning at the C-terminals with the N-terminals of the two peptides each forming one of the globular heads (Katz, 1970).

Tryptic digestion of skeletal myosin appears to occur via a rapid hydrolysis of peptide bonds in a non-helical region of the molecule followed by a slower reaction representing hydrolysis in an α-helical region, with the digest conditions yielding different lengths of the HMM "tail". In cardiac myosin tryptic digestion appears to proceed as the slow reaction, suggesting possible differences in secondary or tertiary structure or different amino acid sequences in the susceptible regions of the molecule (Katz, 1970).

The hydrodynamic properties of HMM indicate that this fragment exists primarily in globular form to which is attached a short, helical rod (Katz, 1970). Papain cleavage or brief tryptic digestion of HMM yield the globular HMM subfragment 1 (HMM-S_1) and the short rod-like portion, subfragment 2 (HMM-S_2). The HMM-S_1 has two globular heads and retains the solubility and enzymatic properties of the entire HMM having a molecular weight of 115,000 d per head while the S_2 fragment remains soluble at low ionic strength but exhibits no enzymatic properties and has a molecular weight of approximately 60,000 d (Mannherz and Goody, 1976; Lowey et al., 1969).

Anti-body staining techniques against either HMM-S_1 or HMM-S_2 revealed that these proteolytic fragments participate as the cross-bridge projections observed in electron micrographs (Mannherz and Goody, 1974). Huxley (1969) suggested that the site(s) of papain cleavage on the HMM fragment represents two regions of reduced structural rigidity and permit the S_2 portion and S_1 heads to function as hinges allowing them to swing out, swivel and attach to actin thus fulfilling the myosin head's role as a force-generating transducer in contraction.

Light Chains

Concentrated dissociating agents such as 6-8 M urea or 5 M guanidine disrupt the myosin molecule into 2 fairly homogenous heavy polypeptide chains (HC: 200,000 d each) plus 2 to 4 low molecular weight subunits termed light chains (LC) that are non-covantly linked to the main polypeptide chains of the globular head regions (Lowey and Risby, 1971).

In fast skeletal muscle, three electropheretic components have been identified on SDS (sodium dodecyl sulfate) polyacrylamide gel electrophoresis indicating differences in net charge and molecular weight. The three light chains (LC) were separated by chromatography and thiol sequences analysis revealed chemical similarities between two (Weeds and Lowey, 1971). The third component can be removed from myosin by treatment with DTNB (5,5^1-dithiobis-2-nitrobenzoic acid), without loss of ATPase activity; hence its classification as non-essential (DTNB-LC). The molecular weight of the DTNB-LC is given as 18,500 d and isotope dilution studies have shown that two moles of DTNB-LC are attached to one mole myosin or HMM giving a molar ratio of 2:1 (Weeds and Lowey, 1971). HMM-S_1 has only half the expected number of DTNB-LC indicating that the LC may be susceptible to papain digestion (Mannherz and Goody, 1976). In addition, myosin has been shown to bind 2 moles of Ca^{2+} (free calcium) with high affinity to sites associated with the DTNB-LC (Marimoto and Harrington, 1974). It has been suggested that the divalent metal ions afford some protection of the DTNB-LC from tryptic or chymotryptic attack due to their S_1-S_2 region proximity releasing the HMM fragment, whereas papain may cleave at a different site unaffected by divalent ions and therefore releases the S_1 and some DTNB-LC (Bagshaw, 1977). Papain may in fact cleave at lysine 82 of the heavy chain near the myosin active site (Ramirez et al., 1979).

The two chemically-similar light chains belong to the essential class with molecular weights according to SDS gel electrophoresis of 25,000 d (LC_1) and 16,000 d (LC_3). They can be removed from myosin under various conditions including acylation, treatment with denaturing agents, or exposure to an alkaline pH of 11, with subsequent loss of ATPase activity and are referred to as the alkali light chains (Weeds and Lowey, 1971; Mannherz and Goody, 1976). Alkali LC_1 differs from LC_3 in its additional 41 amino acid residues at the N-terminus plus five other amino acid substitutions (Mannherz and Goody, 1976). The stoichiometry of the alkali light chains determined from isotope dilution studies shows that myosin and HMM contain 2 moles of the essential LC, while HMM-S_1 retains only a single alkali LC non-covalently bound (Weeds and Lowey, 1971). The ratio of LC_1:LC_3 in rabbit psoas muscle appears to be about 2:1 where individual myosin molecules contain either two LC_1 or LC_3 (Weeds et al., 1975), although more recent studies have suggested that an LC_1:LC_3 heterodimer also exists (Lowey et al., 1979). The function of the alkali light chains may involve modification of both ATPase and actin-binding activities since significant differences between LC_1 and LC_3 regarding Mg^{2+}-ATPase activity have been reported (Wagner and Weeds, 1977).

The light chain patterns of cardiac and slow skeletal myosin differ from that of fast skeletal myosin. Treatment of cardiac myosin with DTNB does not release an 18,500 d LC. Gel electrophoresis with and without SDS revealed that cardiac myosin contains only two light chains with molecular weights of 27,000 d (LC_1) and 19,000 d (LC_2) while rabbit red skeletal muscle contains three LC types: LC_1 (27,000), LC_2 (19,000) and LC_3 (29,000) (Frearson and Perry, 1975). Removal of LC_2 in cardiac myosin results in reduced K^+-(EDTA) ATPase activity, increased Mg^{2+}-ATPase activity and a striking increase in actin-activated Mg^{2+}-ATPase activity suggesting an important role of LC_2 in actin-myosin interaction (Malhotra et al., 1979). Cardiac myosin has been shown to bind to only one of the two HMM-S_1 heads released by chymotryptic digest and may in fact bind directly to a short 5,000 d polypeptide chain associated with one of the S_1 heads at or near the S_1-S_2 link region (Kuwayama and Yagi, 1980).

The 18,000 to 20,000 d light chains of fast skeletal and cardiac muscle from rabbits display considerable amino acid homology with the exception of serine and valine contents (Frearson and Perry, 1975). Homologous LC_2 components of heart, smooth muscle, skeletal muscle, vertebrate, invertebrate and non-muscle cells all display the ability to bind divalent metal ions (acting as Ca^{2+} receptors) and act as substrates for phosphoryl transfer reactions involving specific kinases and phosphatases (Holroyde et al., 1979). A highly specific myosin light chain kinase (MLCK) has been identified which catalyzes the transfer of the y phosphate of ATP to a single serine residue in the DTNB or LC_2 component of cardiac or skeletal myosin (Perrie et al., 1973; Pires et al., 1974; Frearson and Perry, 1975) while a specific protein phosphatase dephosphorylates the residue (Perry et al., 1975). MLCK also appears to require Ca^{2+} in a regulatory role (Pires et al., 1974). The covalent phosphate content of heart and skeletal LC_2 (or P light chain) has been shown to turn over and change in beating hearts (Frearson et al., 1976) and in live skeletal muscle (Barany and Barany, 1977). Freeze clamping of beating hearts revealed levels of 0.3 to 0.4 mole phosphate per mole of LC_2 (Holroyde et al., 1979) which is similar to levels found in frog white skeletal myosin (Barany and Barany, 1977) and in rabbit fast muscle (Stull and High, 1977).

Active Site

It has been established that the enzymatic and actin-combining properties of myosin reside in the HMM-S_1 head region composed of the NH_2 terminals of the two heavy chains and light chains. The exact nature and location of the active site has not yet been elucidated. Part of the obscurity is attributable to the paucity of information regarding heavy chain primary structure while similar information on the light chains has become available. Elzinga and Collins (1977) isolated a 92 amino acid fragment from the head of the myosin molecule from a CNBr (cyanogen bromide) digest of rabbit fast skeletal muscle. The presence of the unusual amino acid N^{τ}-methylhistidine with its strong Mg^{2+} (free magnesium) affinity at position 69 of the 92-residue P_{10} peptide, as well as the presence of two cysteinyl residues whose -SH (sulfhydryl) group alkylation altered the enzymatic properties of HMM and myosin (Seidel, 1973), suggested that the P_{10} peptide may form some or all of the myosin ATPase active site. Recent studies indicate that P_{10} is located on S_1 proximal to S_2 in the proposed hinge region of the myosin heavy chain (Lu et al., 1978). The reactive thiol groups SH_1 and SH_2 of cysteines 21 and 11 respectively on P_{10} near the hinge region could be modified by MgATP presence as could an additional SH_3 located on a small peptide also at the hinge region (Schaub et al., 1978). The apparent proxmity of these SH groups to the myosin active site and to the hinge region suggest that the active site is also proximal to the hinge region (Ramirez et al., 1979).

Ramirez et al. (1979) developed a model for a main element of the skeletal myosin active site which relates directly to the P_{10} peptide. MgATP, the eight-membered cyclic substrate, fits tightly into a 16 amino acid pocket on P_{10} and directly interacts with 7 of 16 amino acids. An important feature of this model is the role of N^{τ}-methylhistidine at position 69 which could function as a donor ligand to Mg^{2+}. In cardiac myosin and other myosins lacking the methylated amino acid, the Mg^{2+}-coordination role could be assumed by histidine 69. The other proposed amino acids in the model are: 1) tyrosine 72 which may attach to MgATP via Mg-bound H_2O, 2) histidine 76 which donates a proton to the y phosphate of ATP, 3) lysine 78, which binds electrostatically to the β phosphate of ATP, 4) phenylalanines 80 and 81 which flank the ATP purine ring, and 5) asprtate 66 which forms a hydrogen bond to the 6-amino group of adenine. The tertiary structure conferred on the peptide by primary structure would permit the involvement of other functional groups.

Heavy chain functional groups which have been implicated in ATPase activity include the sulfhydryl groups SH_1 and SH_2 already mentioned (Srivastava and Wikman-Coffelt, 1980), reactive lysyl residues in the S_1-S_2 region (Hiratsuka, 1981) and a reactive carboxyl group (Korner et al., 1982). With regard to the SH_1 and SH_2 groups, modification may in some way affect the coordination sphere of the magnesium. These two groups are part of a long, moveable chain bound at one end by residues 1 to 9 of the helix and at the other by the 16 amino acids implicated in the active site. The presence of glycine residues in the chain permitting pivotal movement could conceivably allow direct or indirect coordination with Mg^{2+} possibly by its attachment to other ligands (i.e., tyrosines 38 or 31) (Ramirez et al., 1979).

Various labelling techniques have lead to the suggestion that lysyl residues located in the S_1 fragment may be capable of influencing ATPase and/or actin-binding activities. Muhlrad et al. (1981) carried out trinitrophenylation studies on the reactive lysyl residues (RLR) located on the 27 fragment of the N-terminal peptides of atrial and ventricular heavy chains. They found 1.7 to 2.0 RLR's per molecule in myosins and HMMS from rabbit skeletal and sheep atrial and ventricular muscles. At least one RLR was reported near the SH_1 thiol group in the three-dimensional structure which was reflected in functional similarities of increased Mg^{2+}-ATPase responses to RLR trinitrophenylation and SH_1 aklylation. RLR modification also reduced K^+-(EDTA) ATPase activity. The labelling of cardiac RLR's in the S_1-S_2 region with Mns-Cl (n-methyl-2-anilino-6-naphthalene-sulforyl chloride) did not affect CA^{2+} or K^+-(EDTA) ATPase activity but did reduce actin-binding affinity of the cardiac myosin (Hiratsuka, 1981). Muhlrad et al. (1981) suggested that different lysyl residues were involved in the two different labelling techniques.

Carbondiimide modification of a carboxyl group from cardiac myosin reduced Ca^{2+}- and K^+-(EDTA) ATPase activities, as in skeletal muscle, however was not protected by nucleotide presence. This suggests that the carboxyl group is not located on the bindery site of ATP or ADP and that the enzyme nucleotide complexes of skeletal and cardiac myosins may be structurally different. However, one carboxyl group is essential for active conformation of both cardiac and skeletal myosins (Korner et al., 1982).

Myosin Isoenzymes

Muscle type specificity of myosin has been ascribed to differences in both light and heavy chains. Immunological differences have been reported for heavy chains in skeletal fast and slow and cardiac myosin (d'Albis et al., 1979) while electrophoretic differences of some light chains have also been found (Hoh et al., 1978). The precise contribution and nature of LC and HC differentiation to myosin polymorphism is not yet clear but appears to differ between muscle types.

Hoh et al. (1976), in attempting to resolve the heterogeneity of skeletal-muscle myosins observed in physiological and biochemical properties, solubilized chicken posterior latissimus dorsi (PLD) and anterior latissimus dorsi (ALD) muscles in pyrophosphate buffers and compared their electrophoretic mobilities on polyacrylamide gels. The results demonstrated that fast PLD myosin could be resolved into three electrophoretic components while slow ALD had two components, supporting the concept of inherent heterogeneity of myosin. Light chain analysis in SDS polyacrylamide gels revealed that the three PLD forms were attributable to homodimer myosins containing either two alkali LC_1, two LC_3 and possibly a heterodimer of one LC_1 and LC_3 each (Hoh, 1978). This is supported by the observation of non-integral stoichiometry of alkali light chains observed in rabbit skeletal myosin (Weeds et al., 1975). The slow ALD myosin contained only two LC components LC_1 and LC_2 in a 1:1 ratio indicating that slow muscle myosin heterogeneity may reside in the heavy chains (Hoh, 1978). Lowey et al. (1979) substantiated the existence of a fast myosin heterodimer by using specific N-terminal sequence antibodies of LC_1 or LC_3. Similarly, Bechet et al. (1982) obtained 35% heterodimer in rabbit fast skeletal muscle. d'Albis et al. (1979) demonstrated that the proteolytic digestion patterns of chicken PLD were essentially identical while obvious differences were observed in slow skeletal and cardiac myosin heavy chains. Therefore, HC variation may contribute more to slow and cardiac myosin polymorphism while the alkali light chains differentiate the fast skeletal myosin isoenzymes (d'Albis et al., 1979; Hoh, 1978).

Electrophoretic analysis in pyrophosphate gels of intact myosin from rat myocardium revealed the presence of five distinct components;

two in atrial myosin (A_1, A_2) and three in ventricular myosin (V_1, V_2, V_3) (Hoh et al., 1978). Analysis of Ca^{2+}-activated ATPase activity in the gels gave similar values for A_1, A_2 and V_1, lowest activity in V_3 and intermediate activity in V_2. Light chain contents were similar between atria and ventricles, each having LC_1 and LC_2 types. The LC_2 species of atria had a molecular weight of 22,000 d as compared to that of ventricular LC_2 at 19,000 d. The V-LC_2:V-LC_1 ratio was 1:1.14 ± 0.11 while that of A-LC_2:A-LC_1 was 1:2.34 ± 0.35. The functional significance of this difference is not clear. The occurrence of three ventricular myosins could not be attributed to variation in the relative distribution of light chains nor could light chain content relative to heavy chains explain the polymorphism. Thus, the heterogeneity is thought to reside in the heavy chain structure (Hoh et al., 1978). Reciprocal changes in the acidic and basic residues of heavy chains have been reported in euthyroid and hypothyroid ventricular myosin, reducing the net negative charge on the heavy chains of hypothyroid myosin (Yazaki and Raben, 1975; Hoh et al., 1978). Thyroid hormone manipulation has produced shifts in ventricular myosin isoenzyme patterns which may be explained by the above where the excess of positive charges correspond to V_3 heavy chains (Hoh et al., 1978). Comparison of CNBr peptides of rat ventricular myosin heavy chains and two-dimensional peptide mapping further support the existence of three electrophoretically-distinct forms of ventricular myosin differing in heavy chain structure (Hoh et al., 1979). A significantly greater content of the amino acid methionine and significantly less arginine are found in V_1 as compared to V_3 on a mol/mol HC basis (Hoh et al., 1979). Zak et al. (1982) also report a different primary structure of heavy chains obtained from a partial chymotryptic digest of V_1 and V_3 isomyosins. V_2 appears to be a distinct hybrid isoenzyme.

The distribution of the ventricular isomyosins is somewhat variable across species. The V_1, V_2 and V_3 components have been electrophoretically identified in rat, rabbit, mouse, pig and possibly beef ventricles while only V_3 has been identified in guinea pig, canine and human ventricles. In general it seems that larger, slower beating hearts may possess predominantly the V_3 isoenzyme with a lower Ca^{2+}-ATPase activity. The lack of other isoenzymes in these muscles may also stem from a methodological problem as immunological studies have detected the V_1 isoenzyme as well as V_3 in guinea pig hearts. More conclusive results may be obtained by examination of electrophoretic mobility, reactivity with specific antibodies and Ca^{2+}-ATPase activities in combination (Clark et al., 1982).

Thick Filament Organization

Aggregated myosin molecules at low ionic strength give rise to thick filaments arranged in a head-to-head manner maintaining opposite polarity in the filament center. X-ray diffraction and electron microscope studies have shown that the globular cross-bridges project from the filament in a helical arrangement with an axial spacing of 145 Å (Huxley and Brown, 1967). Two types of interactions of myosin molecules are required for this assembly: molecules at the filament centers point in opposite directions and are bound together by interaction between the rod sections while molecules used to extend the length of the filament attach by interaction with molecules pointing in the same direction (Mannherz and Goody, 1976). Huxley and Brown (1967) proposed a double-stranded α helix of cross-bridges around each thick filament. Squire (1972) however has suggested a three- or four-stranded model while Marimoto and Harrington (1974) support a four-fold rotational symmetry. Haselgrove (1980) demonstrated through X-ray diffraction and modelbuilding calculations that the cross-bridges in relaxed muscle are wrapped around the filament rather than projecting normal to the filament surface. The diffraction pattern suggests that each cross-bridge has 2 heads pointing along the helices with opposite screw senses; that is, the myosin heads are twisted the same way around the filament but tilt in opposite directions. In frog sartorius muscle, the 12 nm-long heads were tilted at +30° and -30° to the filament axis and were skewed around the filament by about 60 to 90° to wrap around the filament (Haselgrove, 1980).

THIN FILAMENTS

The thin filaments in striated muscle contain the proteins actin, tropomyosin and troponin in a molar ratio of 7:1:1 as originally proposed by Ebashi and Endo (1968). A number of biochemical, antibody labelling, X-ray diffraction, electron microscopy and optical diffraction studies have further defined the structural relationships of thin filament proteins (Mannherz and Goody, 1976). The double helical arrangement of actin polymers gives the thin filament the typical appearance of two strands of pearls wound around each other when viewed in negatively stained electron micrographs (Hanson and Lowey, 1963). The rod-shaped tropomyosin molecule is situated in the grooves between the two actin polymer strands, while the troponin complex is associated with both actin and tropomyosin at a repeat distance of 385 Å along the thin filament (Ebashi and Endo, 1968).

Actin

Actin constitutes approximately 60% of thin filament protein and is a single chain protein having 376 amino acid residues (Elzinga et al., 1973). Since actin is a highly-conserved protein across species and tissue types, cardiac and skeletal actin structure are homologous (Mannherz and Goody, 1976). Finlayson et al. (1981) reported that only 1% of the amino acid sequence between skeletal and cardiac actin differs and involves a single residue inversion and two substitutions with no overall change in the actin monomers.

Actin exists in two states: globular G-actin which is Ha monomer and is stable in solutions of low ionic strength and fibrous F-actin, which is a G-actin polymer formed upon addition of neutral salts. The actin G to F transformation or polymerization is accompanied by a marked increase in the viscosity of the solution (Katz, 1970). The molecular weight of native G-actin according to its amino acid sequence is 41,785 d, and for G-actin containing one bound ATP and one Ca^{2+} as prosthetic groups is 42,300 d (Elzinga et al., 1973). In the formation of the actin polymer, the actin monomers (55 Å in diameter) are arranged in a genetic helix of 59 Å-pitch, which can be incorporated into a helix of 2 x 370-385 Å pitch giving the characteristic thin filament appearance (Hanson and Lowey, 1963).

Skeletal and cardiac actins exhibit similar physicochemical properties. The globular shape of G-actin is reflected by its low viscosity and also appears to have some degree of asymmetry based on amino acid content (Katz, 1970).

The sequencing of skeletal muscle actin reveals the presence of several potentially-important features regarding tertiary structure and function. The N-acetyl aspartic acids on the N-terminus and C-terminus are both highly charged. The appearance of the unusual amino acid N^{τ}-methylhistidine at position 73 suggests a specific biologic function especially in light of a similar presence near the myosin active site. The occurrence of 19 proline residues supports a low α-helix content in

G-actin of approximately 30% (Elzinga et al., 1973). The slight asymmetry of G-actin has been attributed to a single tyrosyl residue at position 53. One tyrosine per actin monomer is required for polymerization indicating that this amino acid is associated with a structurally-distinct region of G-actin (Bender et al., 1974).

Skeletal and cardiac actins contain five cysteine residues with sulfhydryl (SH) groups that can be labelled with a number of reagents in order to gain insight into their microenvironments and accessibility and involvement in actin polymerization (Elzinga et al., 1973; Mannherz and Goody, 1976). Cysteine-10 (cys-10) was labelled by iodoacetate in both G- and F-actin while iodoacetamide labelled cys-10, cys-283 and cys-373 in both actin states which indicated that the C-terminus of actin is more non-polar. Cys-283 and -373 do not seem to be involved in polymerization or nucleotide binding since these processes were not influenced by inactivation of the SH groups. Treatment by 6 M guanidine exposed only cys-156. In the actomyosin complex cys-373 is not accessible although its carboxylation does not impair actin-myosin interaction. While organomercurial compounds at cys-10, -283 and -373 reduce polymerizability there is not parallel reduction in nucleotide release suggesting that the SH groups on actin may only serve a subsidiary functional role in actin polymerization (Mannherz and Goody, 1976).

G-actin contains a single site for a nucleotide and preferentially binds ATP (adenosine triphosphate) with a binding constant of 10^{10} M^{-1} (Barden et al., 1980). In addition, it also possesses a single, high-affinity divalent cation binding site which is normally occupied by Mg^{2+} because of its high intracellular concentration. Binding of Ca^{2+} to this site appears to influence polymerization kinetics (Barden et al., 1980; Wegner, 1982). The affinity for Ca^{2+} is about 4 times that for Mg^{2+}

(Katz, 1970). Binding of both a nucleotide and a divalent cation is necessary for G-actin polymerization. Earlier evidence suggested that the the nucleotide and cation were bound at or near a single site on G-actin since the removal of one parallelled loss of the other. However, Barden et al. (1980), using high resolution proton nuclear magnetic resonance (PNMR) and electron paramagnetic resonance (EPR) spectroscopy, demonstrated that the sites are separated by a distance of 16 Å and that the spectra were not altered by actin polymerization in this regard. Affinity-labelling of the nucleotide binding site on actin after blocking cys-373 indicated that a single tyrosine residue may be responsible (Barden et al., 1980). Occupancy of the cation binding site is a requisite for strong nucleotide binding (Mannherz and Goody, 1976).and it appears that the cations may have a more specific role in stabilizing the F-actin structure besides as a compensator of negative protein charges and in reducing electrostatic repulsion between the monomers (Zechel, 1981). Nucleotide phosphorylation does not appear essential for polymerization; however polymerization rate is enhanced when G-actin is bound to ATP rather than ADP (Katz, 1970).

The formation of actin filaments has been shown to result from nucleation (aggregation of a few monomers) and consecutive binding of actin monomers to the ends of these filaments in a process termed elongation (Wegner, 1982). The filaments associate in an end-to-end manner and the polymerization kinetics are of an autocatalytic nature (Wegner, 1976; 1982). The resulting polymer formed by secondary bonding between the globular protein molecules suggests that the F-actin molecule should be flexible (Oosawa, 1980). The results of light-scattering, electron microscope, fluorescence and thermal binding studies all portray a uniform, semi-flexible, F-actin filament (Nagashima and

and Asakura, 1980; Oosawa, 1980). The flexibility is influenced by the presence of different cations, especially Ca^{2+} and Mg^{2+}. This F-actin flexibility is thought to be of considerable consequence in its interaction with myosin and ATP (Oosawa, 1980).

Tropomyosin

Tropomyosin (Tm) is a rod-shaped, polar molecule approximately 410 Å long and 20 Å wide and constitutes 10 to 12% of the myofibrillar protein in skeletal muscle (Mannherz and Goody, 1976; Katz, 1970). It is a highly helical dimer and is considered the smallest and simplest of the α-group of fibrous proteins which also include the rod portion of myosin, α-keratin and paramyosin (Sodek et al., 1978). Perry and Corsi (1958) first suggested the coexistence of tropomyosin and actin in a single structure of the myofibrils upon observing the coextraction of the two proteins from muscle. Since then, Tm has been shown to lie in the groove of the actin helix, associates with actin filaments in a stoichiometric ratio of 1:7 (actin), and is intimately involved in the calcium regulatory system of skeletal and cardiac muscle (Cummins and Perry, 1974).

The physicochemical properties of skeletal and cardiac muscle Tm differ little and have been extensively studied due to its ready solubility in aqueous solutions (Katz, 1970; Sodek et al., 1978). In concentrated salt solutions the molecular weight of Tm is about 68,000 d. Under reducing conditions, denaturing agents such as concentrated urea or guanidine dissociate the protein into two polypeptide chains of approximately 34,000 d accompanied by a reduction in α-helical content from a value close to 100% to almost zero as estimated by optical rotatory dispersion and circular dichroism properties. In dilute salt solutions (0.1 MKcl) polymerization of Tm molecules in an end-to-end manner forming long filaments is facilitated. Under other conditions, in the presence of divalent cations, Tm molecules form highly-ordered fibrous aggregates, tactoids or paracrystals, which under the electron microscope after staining with uranyl salts, shows a detailed banding pattern with a period of 385 Å (Sodek et al., 1978).

The two subunits released by denaturing agents are arranged in a two-stranded coiled-coil in the Tm molecule. Electrophoretic studies have revealed the presence of two distinct chains in rabbit skeletal muscle Tm termed α and β chains which have also been observed in skeletal Tm of other mammalian, avian and amphibian species (Cummins and Perry, 1974). The α chain predominates with a ratio of 4 α:1 β giving rise to two dimer molecules, αα and αβ (Mannherz and Goody, 1976; Lehrer, 1975). The two polypeptide chains differ slightly in their amino acid compositions, molecular weights and immunological properties. Wegner (1980) reported that the α and β chains differed by 39 amino acids, 23 of which were situated at the molecular surface. In addition, there were 2 amino acid substitutions in the last 9 residues of the C-terminal where Tm molecules form a polar, end-to-end contact with the N-terminal of an adjacent Tm molecule bound along the actin filament. The same C-terminal residue (isoleucine) has been found for a number of tropomyosins while the last 140 residues appear to be highly conserved (Mannherz and Goody, 1976). Using light scattering techniques and analyzing the binding curves of Tm affinity for actin and end-to-end affinity with adjacent Tm molecules, Wegner (1980) found very little difference between the α and β subunits suggesting that their structural disparities may not influence function.

The major difference between α and β subunits is found in their thiol content where α chains contain one cysteine residue (SH group) and β chains contain two per 34,000 g or per chain approximately (Cummins and Perry, 1974). In both α and β subunits one cysteine is located at position 190. Thiol content is mainly responsible for the effect of Tm on the Ca^{2+}-stimulated ATPase activity of troponin-free desensitized actomyosin (DAM) but bears little effect in the presence of troponin and therefore thiol content differences between α and β chains may not be physiologically significant (Cummins and Perry, 1974).

The α and β subunits of skeletal muscle also demonstrate very different antigenicity where the antibody raised against rabbit skeletal α-Tm was found to be species non-specific while that of β-Tm was species-specific (Cummins and Perry, 1974).

Skeletal-cardiac tropomyosin comparisons and cardiac tropomyosin species differences were examined by Leger et al. (1976). Cardiac Tm from small animals such as rabbit, guinea pig, rat and dog contain 2 SH/mole and were resolved into one band on SDS and acid urea electrophoresis and into two bands on alkaline urea electrophoresis, the molecular weight of each band ~ 32,000 d. These tropomyosins bore similar M.W., thiol content and charge as the rabbit skeletal α-Tm suggesting that these cardiac Tm's were of the αα type. Isoelectric focusing of the two alkaline urea bands demonstrated the existence of a_1 and a_2 and β_1 and β_2 components in rabbit skeletal Tm. Chicken heart Tm gave only one band but contained 4 SH/mole. Its molecular weight was similar to that of rabbit skeletal α-Tm on SDS and had the same charge on acid urea electrophoresis, however, its charge on alkaline urea electrophoresis and its thiol content were closer to that of β-Tm. Two bands, α and β, were observed on SDS gel electrophoresis for pig, sheep and human cardiac Tm (MN: 32,500-36,000 d) and gave 2.6, 2.4 and 2.4 Sh/mole respectively. The α components from sheep skeletal and pig and sheep cardiac Tm were more positively charged than the rabbit α-Tm component. Human Tm components showed similar charges as the rabbit skeletal Tm chains. Further studies comparing α-Tm in rabbit cardiac and skeletal muscles demonstrated homology in primary structure by cyanogen bromide and tryptic peptide analysis, electrophoretic mobility, immunological properties and NH_2-terminal analysis (Cummins, 1979; Lewis and Smillie, 1980). In addition, frog and rabbit skeletal and cardiac α-Tm have been found to exist in part in a phosphorylated form, the significance of which is unclear (Lewis and Smillie, 1980).

The polymorphic nature of vertebrate tropomyosin appears to be related to muscle type. Cummins and Perry (1974) and Leger et al. (1976) among others have noted an inverse correlation between the relative proportion of the β-component and the contraction speed of the particular muscle. In skeletal muscle, red, slower fibers contain a greater percentage of β-chain; β-chain proportion is also directly related to body size. In cardiac muscle the β-component proportion seems to reflect inversely the speed of contraction and directly the heart size since β-subunits are absent in smaller, rapidly-beating hearts but may constitute up to 20% of total Tm in larger, slower hearts. Tropomyosin heterogeneity may therefore influence the function of myosin ATPase since ATPase activity determines a muscle's speed of shortening (Leger et al., 1976). The biological properties with regard to Tm's influence on Ca^{2+}-stimulated and Mg^{2+}-stimulated ATPase activities differed only in the extent of influence but were similar in direction amongst a number of Tm sources (Leger et al., 1976).

The elucidations of the complete amino acid sequence of rabbit skeletal α-Tm (Sodek et al., 1978; Stone and Smillie, 1978) and its analysis (McLachlan and Stewart, 1976a, b) has revealed the presence of several periodic and non-periodic features which can be correlated with the stabilization of its coiled-coil in a parallel, non-staggering arrangement, with its head-to-tail aggregation and with its interaction with F-actin and troponin-T (Th-T) (Smillie et al., 1980). The structural analysis of Tm undertaken by McLachlan and Stewart (1976a) provided a fundamental understanding of its molecular organization and function based upon earlier electron microscope and sequence studies and on Crick's model of knobs and holes for the intramolecular stabilization of coiled-coil molecules. In the McLachlan-Stewart proposal, a Fourier analysis of the distribution of different types of amino acids in the Tm sequence showed strong 14th order peaks in the profiles of both negatively charged and non-polar amino acids with a period of 19 2/3 residues or 29.3 Å and an overall repeat length of 275 ± 2 amino acids, slightly less than the sequence length of 284 residues. The analysis assumed a supercoil structure with two parallel α helices in register coiled with a pitch of 137 Å. End-to-end molecular overlap of 8 or 9 residues would permit the continuation of the periodicity throughout the Tm polymer length. Periodic features were more pronounced on the outer surface of the molecule.

The amino acid sequence was divided into 14 bands which each have a narrow zone of net positive charge and a broader band of net negative charge. Overlapping every positive zone is a region of hydrophobic residues with at least one non-polar group on the outer surface. The alternating negatively-charged and non-polar zones represent regions where TM molecules may be only loosely connected to actin. The acidic groups in the negative zones may attach to actin via numerous salt bridges which might make direct links to basic groups on actin or bridge indirectly to acidic groups via intervening Mg^{2+} and/or Ca^{2+} ions. Both types of bridges would form rather flexible links compatible with noted irregularities in the periodic spacing of acidic groups and would allow easy conversion between the "on" and "off" states of tropomyosin's position on actin. Non-polar groups in the positive zones could contribute to the positioning of Tm on actin in the two states. The positive regions appear to be involved in the actin binding sites. McLachlan and Stewart (1976a) base this on two observations. Anomalies in charge distribution and periodicity occur in the overlap regions at filament ends where distortion of the molecule would be likely to disrupt actin binding and also in the Tn-T binding regions; both of these regions occur in negative zones while no major disruptions are noted in the positive zones.

In the thin filament, the 137 Å pitch of the supercoil would make 7 half-turns relative to the twisted actin helix along a 385 Å length such that a pair of adjacent bands on Tm would be oriented equivalently with respect to a pair of actin monomers 28 Å apart. Therefore, the bands were separated into two alternating series, α and β, each having one positive and one negative zone and every pair of bands repeating approximately every 39 1/3 residues, which appears to be an adaptation to the spatial periodicity of the actin helix. In the McLachlan-Stewart model, the 7 α and 7 β bands represent alternate sets of sites which bind equivalently to complimentary groups of sites on 7 actin monomers in the "relaxed" and "active" states respectively. Zones 1 α and 7 β both have unusually well-developed non-polar outerfaces which could strengthen the binding to actin near the ends. On passing between adjacent α and β bands, the supercoil twists 90° relative to actin, therefore a quarter rotation of the entire Tm molecule would allow a highly cooperative switch mechanism in regulating actin-myosin interaction. Another feature of this 7th order periodicity is the distribution of small non-polar amino acids along the helix which may account for the presence of flexible regions every 55 Å where a bend about the broad face of the supercoil is facilitated (McLachlan and Stewart, 1976a).

Subsequent research has lent support to McLachlan's and Stewart's proposal (1976a) and further explained the periodic features and kinking characteristics of the Tm molecule. The complete amino acid sequence did not demonstrate periodicity of specific amino acids but did support periodicity of types of amino acid groups (Stone and Smillie, 1978). Smillie et al. (1980) suggested that the α bands represent homostrand interactions with actin while β bands, the heterostrand interactions. Three-dimensional image reconstruction of the Tm filament showed that the Tm strand rather than running in a smooth curve in the actin groove, is parallel to the actin filament axis in the regions of homostrand interactions and runs obliquely between these regions (Wakabayashi et al., 1975). A regular 7-fold periodicity was further demonstrated by plotting the average helix parameter against peptide bond number and revealed distinct maxima and minima at intervals of close to 40 residues. The maxima appear to correspond to non-polar positive zones of β regions, while the minima represent non-polar positive zones in the α bands. The greater flexibility of the less-ordered minima could confer local structural instability to Tm and partially account for the periodic binding (Smillie et al., 1980). Further investigation of these flexible regions through various digestive procedures led Pato and Smillie (1981a) to conclude that stabilization of the coiled-coil structure is attributable largely to the repeating pattern of polar and non-polar residues with a smaller contribution from electrostatic interaction. Their research also showed that fragments from the COOH terminal were less thermally stable and exhibited less-distinct periodicity than NH_2-terminal half fragments. This was suggested to reflect considerable variation in the nature and affinity of actin-binding sites along the Tm molecule as well as the imposition on the COOH-terminal third of the structural requirements for the binding of Tn-T (Pato and Smillie, 1981a).

Evidence that tropomyosin may exist in different conformational states has accrued from cross-linking and structural stability studies. The parallel, non-staggered arrangement of Tm chains gives rise to an intramolecular disulfide bond between the cys 190 residues of both chains (Lehrer, 1975). In the 30-40°C range Tm cross-linked at cys 190 is partially unfolded whereas uncross-linked TM remains largely α-helical under these conditions (Walsh and Wenger, 1980). It was further demonstrated that the stability of actin-Tm complexes was dependent upon the oxidative state of cys 190 where uncross-linked Tm bound more tightly to actin filaments than cross-linked Tm. That small changes in structure and flexibility of Tm can alter interaction with actin now seems apparent (Walsh and Wegner, 1980). O'Hara et al. (1980) demonstrated additional cross-linking between Lys 5 and Lys 7 of the two Tm strands which appears to influence head-to-tail aggregation of Tm molecules (Stone and Smillie, 1978). Edwards and Sykes (1980, 1981) utilized PNMR techniques (proton nuclear magnetic resonance) to analyze the cooperative binding curves of histidine residues 153 and 276. Three conformational states associated with thermal unfolding of the peptide chains around these residues were noted: fully-folded, partially-unfolded and effectively unfolded (only 45% helicity). These states differed in proton affinities and/or chemical shifts and appeared to be in slow exchange with one another. Positive cooperativity between the two histidine residues was reported with His-153 being the most responsive of the pair. Polymerization of Tm enhanced the cooperativity and favours the low affinity conformation in which the histidine residues have a lower affinity for protons. Formation of a cross-link between cys 190 of both strands destabilizes the middle region of Tm and contributes to conformational changes at His-153 (Edwards and Sykes, 1981).

Dramatic viscosity increases upon combination of Tm and troponin first indicated interaction of the two proteins (Pato and Smillie, 1981). Evidence for the binding site on tropomyosin for troponin has been largely derived from electron microscope examination of fibrous magnesium paracrystals of the protein stained with uranyl acetate (Stewart and McLachlan, 1976; Stone and Smillie, 1978). These show a characteristic pattern of dark and light bands with two dyed (stain-excluding) axes, which have been interpreted in terms of a repeating array of antiparallel molecules with one dyad axis within a 5 Å of leucine-197 (Stewart and McLachlan, 1976). Troponin has been shown to bind over a 100 Å region centered close to this dyad axis. Pairs of troponin subunits connected to oppositely directed pairs of Tm filaments may contribute to this stain-excluding region where the centers of the individual troponin units are within 25 to 30 Å of the dyad (Stewart and McLachlan, 1976). McLachlan and Stewart (1976) pointed out that the region of sequence between residues 197 and 217 is atypical in being relatively devoid of acidic residues, in having a significantly high concentration of uncharged polar groups and a large hydrophobic surface on the broad face of the coiled-coil. Additionally, this region is flanked by a concentration of acidic groups in sections 192-197 and 218-224. Thus, the non-polar and H-bonding groups could provide highly-specific, closely-packed contacts protected from surrounding H_2O while clusters of negative charges on either side could form ionic bridges to basic groups on Tn-T. Biochemical and structural evidence support the location of the Tn-T binding site in this region. Jackson et al. (1975) demonstrated the binding of Tn-T residues 70-160 to the Tm independent of Ca^{2+} and Mg^{2+} ions but influenced by ionic strength. Soluble cyanogen bromide fragments of Tn-T (CB_1: 1-151 and CB_2:71-151) have been shown to bind to the COOH-terminal third of TM (Pato and Smillie, 1981b). Both affinity chromatography and gel filtration techniques have provided evidence that fragments from the COOH-terminal third of Tm bind the CB_1 fragment of Tn-T, while NH_2 terminal fragments do not. When fragments of NH_2-terminal and COOH-terminal sequences are combined, a ternary complex with CB_1 is formed under conditions in which the two Tm fragments alone show no interaction. Pato and Smillie (1981b) interpreted these observations as indicating that the binding of Tn-T fragment CB_1, to the COOH-terminal third of the α-Tm molecule enhances head-to-tail aggregation either by transmission of conformational changes to the overlap regions or more directly, by binding at or near these sites. Nagano et al. (1980) and Nagano and Miyamoto (1981) suggest that the most plausible conformation of the specific Tn-T binding residues 71-151 to the Tm coiled-coil is the formation of a triple-stranded coiled-coil which would permit close packing of the two proteins. The rod-like shape of Tn-T and the high α-helical content (80%) of the CB_2 fragment also led Pato an Smillie (1981b) to propose a multi α-helical protein-protein conformation.

Lateral movement of the tropomyosin filament in the actin helix groove of about 5 to 15 Å during contraction and relaxation cycles has been detected through x-ray diffraction and electronmicroscope studies (Parry and Squire, 1973; Seymour and O'Brien, 1980). Parry and Squire (1973) noted that in the relaxed or "off" state, Tm was oriented at 45° to 50° relative to the actin filament axis which appeared very close to the sites of HMM attachment to actin. In the presence of Ca^{2+} during contraction Tm moved to a position at 65° or 70° relative to actin away from the site of myosin interaction. This suggested either a steric blocking of actin-myosin interaction by tropomyosin upon Ca^{2+}-binding to the troponin complex or modification of the local geometry of myosin-binding sites on actin mediated by movement of the Tm filament. Analyzing their filament reconstructions on micrographs, Seymour and O'Brien (1980) found that Tm filaments occupied positions on the opposite side of the actin groove to that of the postulated myosin S-1 binding site casting doubt on the steric-blocking hypothesis. They suggested a mechanism where Tm only partially-blocks the myosin S-1 binding site of actin. Others (Taylor and Amos, 1981) have more fully developed this line of thought which will be discussed under the Models of Relaxation section.

Troponin

The troponin complex of striated muscle is a specialized form of a system that has evolved for the regulation of processes through changes in calcium concentrations (Perry, 1980). Ebashi and Endo (1968) first proposed that the troponin complex, which is localized on the thin filament with a periodicity of 385 Å, is responsible for the transmission of the Ca^{2+} signal to the thin filament. The troponin (Tn) protein complex has a molecular weight of approximately 76,000 d and is composed of one molecule of each of its three subunits named according to their primary function: TnC (calcium-binding subunit), TnI (troponin-inhibiting subunit) and TnT (tropomyosin-binding subunit) (Mannherz and Goody, 1976). The role of troponin was classically demonstrated by Greaser and Gergely (1971) who isolated and characterized the three subunits and reconstructed them into a complex conferring Ca^{2+}-sensitivity on the ATPase enzyme of the tropomyosin-actin-myosin system.

a) Troponin-C (TnC)

The calcium-binding subunit, TnC, of skeletal troponin has a molecular weight of 17,840 d as established from amino acid sequence studies (Collins et al., 1973). Some variation in the molecular weights of TnC from fast, slow and cardiac muscles exist due to conservative differences in primary structure (Perry, 1980). TnC is an acidic protein with a net negative charge of -30 at neutral pH and an isoelectric point of 4.1 to 4.3 (McCubbin and Kay, 1980). The molecule is a single chain of 159 amino acid residues and has been divided into four internally homologous regions of about 30 residues numbered from I to IV beginning at the NH_2-terminus (Gergely and Leavis, 1980). Structural similarities between these regions and homologous regions in the calcium-binding protein paralbumin were noted by Collins et al. (1973; 1974) leading to the assignment of the Ca^{2+}-binding role to this troponin subunit. Within each of the four regions, there is a ten-residue Ca^{2+}-binding loop rich in aspartic and glutamic acid residues whose carboxylate groups act as coordinating ligands for Ca^{2+}, flanked on either side by α-helical segments (Collins, 1974).

Equilibrium dialysis experiments performed on purified skeletal troponin and on TnC alone revealed that 4 mol Ca^{2+} were bound per mol protein and also identified two classes of Ca^{2+}-binding sites (Potter and Gergely, 1975). The two sites of higher Ca^{2+}-Mg^{2+} sites situated in regions III and IV ($K_{app}Ca^{2+} = 2 \times 10^7\ M^{-1}$; $K_{app}\ Mg^{2+} = 5 \times 10^3\ M^{-1}$). The two low-affinity sites located in regions I and II are Ca^{2+}-specific ($K_{app}Ca^{2+} = 3.2 \times 10^5\ M^{-1}$ (Potter and Gergely, 1975; Potter et al., 1976).

It has also been shown that there is an increase of about one order of magnitude in the affinity of all sites for Ca^{2+} upon interaction with TnI.

A comparison of the amino acid sequences of cardiac and skeletal TnC demonstrated conservative differences in all regions except in the Ca^{2+}-binding loop of region I. Van Erd and Takahasi (1976) characterized bovine cardiac TnC as having a molecular weight of 18,459 d and 161 amino acid residues. It is rich in glutamic and aspartic acid and asparagine residues which usually appear in clusters of two or three. The percentage of amino acid substitutions between cardiac and skeletal TnC in each of the four regions was given as: I (residues 1-48) 48%, II (residues 49-86) 24%, III (residues 87-124) 37% and IV (residues 125-161) 30% (Van Erd and Takahashi, 1976). The greater proportion of substitutions in region I of cardiac TnC involving key coordinating ligands led to the suggestion that this region no longer retained its Ca^{2+}-binding ability. Burtnick and Kay (1977) conducted gel filtration studies comparing the maximum binding of Ca^{2+} by rabbit skeletal and cardiac TnC. They demonstrated that while skeletal TnC bound 3.4 ± 0.2 mol Ca^{2+} per mol TnC, cardiac TnC bound only 3.2 ± 0.2 mol Ca^{2+} which declined to 2.9 mol Ca^{2+} per mol TnC in the presence of 2 mM $MgCl_2$. The association constant for Ca^{2+} was given as $7 \times 10^6\ M^{-1}$ for the three proposed sites which were shown to bind Mg^{2+} competitively (Burtnick and Kay, 1977). Two studies of note were able to detect only one class of binding sites on cardiac TnC (Burtnick and Kay, 1977; Stull and Buss, 1978). However equilibrium dialysis experiments (Potter, 1977) clearly demonstrated two classes of binding sites on TnC: two high-affinity Ca^{2+}-Mg^{2+} sites in regions III and IV ($K_{app}Ca^{2+} = 2 \times 10^7\ M^{-1}$) and a single low-affinity site ($K_{app}Ca^{2+} - 5 \times 10^4\ M^{-1}$) in region

II which agreed with earlier structural analysis (Van Erd and Takahashi, 1976). Further support for the existence of two high-affinity and one Ca^{2+}-specific site arose from tyrosine fluorescence and circular dichroism (CD) experiments utilizing a Ca^{2+}-specific electrode sensitive to changes in free Ca^{2+} concentration (Leavis and Kraft, 1978). The CD and fluorescent spectra for cardiac TnC Ca^{2+}-Mg^{2+} sites were virtually identical to that for skeletal TnC and in addition, these sites showed positive cooperativity in binding (Leavis and Kraft, 1978; Potter, 1977). That site I of cardiac TnC was inactive in Ca^{2+}-binding was further supported by Leavis and Kraft (1978). In this regard, Johnson et al. (1980) labelled cysteines 35 and 84 of cardiac TnC with a fluorescent probe responsive to Ca^{2+}-binding at the Ca^{2+}-specific site(s) and reported that a large increase in fluorescence accompanied Ca^{2+}-binding in the region of cys 84 (site II) while no changes occurred in the other labelled SH group in the mutated site I region. The fluorescence change in site II was insensitive to Mg^{2+}, its binding constant for Ca^{2+} being calculated as 4.5×10^5 M^{-1}. As for skeletal TnC, association with TnI increases the affinity of Ca^{2+}-binding sites in cardiac TnC by about ten-fold to 3×10^6 M^{-1} (Johnson et al., 1980).

The two classes of Ca^{2+}-binding sites appear to fulfill different roles in the regulatory process. Briefly, TnC undergoes a large conformational change upon Ca^{2+} or Mg^{2+} binding to sites III and IV which may be important in maintaining TnC in a conformation prerequisite to Ca^{2+}-activation (Potter and Gergely, 1975; Nagy and Gergely, 1979). Ca^{2+}-binding to the Ca^{2+}-specific site(s) is regarded as the event triggering actin-myosin interaction and full activation of the ATPase enzyme (Potter and Gergely, 1975; Holroyde et al., 1980).

A variety of spectroscopic techniques have shown that Ca^{2+}-binding to the high-affinity sites induces large changes in the secondary and tertiary molecular structure of TnC (Potter et al., 1976; Leavis et al., 1978; Levine et al., 1978). Analysis of circular dichroic (CD) spectra revealed that Ca^{2+}-binding with high affinity even in the presence of denaturing agents (6 M urea) induced the formation of two short α-helical segments containing about 9 or 10 amino acids each which explained the increase in α-helical content from approximately 30% in the divalent cation-free protein to 50% on saturation of the Ca^{2+}-Mg^{2+} sites (Nagy and Gergely, 1979). A 78% increase in α-helical content due to Ca^{2+}-binding to the high affinity sites has also been observed (Johnson et al., 1980). Changes in CD and absorbance in the near UV spectrum also indicated that 4 to 5 phenylalanine residues out of 10 are affected by Ca^{2+}-binding to sites III and IV. One of the α-helical segments formed appears on the NH_2-terminal side of site III while the other is located on the C-terminal side of site IV. (Of the eight helical segments located on either sides of the 4 Ca^{2+}-binding sites and lettered A through H starting at the NH_2-terminal, these segments correspond to helices E and H.) The remaining phenylalanine residues are located in segments adjacent to sites I and II and in the C-terminal side of site III which are α-helical in nature without Ca^{2+} (Nagy and Gergely, 1979).

Proton nuclear magnetic resonance ('H-NMR) spectroscopy has been employed to study conformation changes of TnC in solution. Secondary shifts, which depend on H-bonding, nearby carboxyl groups, degree of ionization and the presence of aromatic ring structures, in a number of resonances indicate a Ca^{2+}-induced increase in secondary structure in the high-affinity domains (Levine et al., 1978). The Ca^{2+}-induced broadening of resonances assigned to tysosine, phenylalanine, glutamate and aspartate residues all indicate further structural changes constraining their movement (Levine et al., 1978). Many of these changes have also been observed in tryptic fragments of TnC containing only the Ca^{2+}-Mg^{2+} sites (Leavis et al., 1978). The behaviour of various optical and spin probes attached to residue cys-98 (cysteine) located within the NH_2-terminal helix of region III (helix E) reflect Ca^{2+}-induced conformational changes in the C-terminal half of TnC (Potter et al., 1976; Nagy and Gergely, 1979). The reduction in the reactivity of cys-98 to thiol reagents in the presence of Ca^{2+} also indicates conformational changes (Potter et al., 1976). Other specific residues demonstrating Ca^{2+}-dependent conformational changes include His 125 whose pK is decreased (Levine et al., 1978) and tyr 109 (tyr 111 in cardiac Tn-C) which showed fluorescent increases with Ca^{2+}-binding (Leavis and Kraft, 1978). The rate of tryptic digestion also appears to depend on Ca^{2+} concentration. In the presence of Ca^{2+}, cleavage of TnC resulted in the formation of two large peptides containing either regions I and II (residues 9-84) or regions III and IV (residues 89-159), while in the divalent cation-free environment, cleavage occurred more rapidly and at several sites previously protected within the conformation (Grabarek et al., 1981). Potentiometric titration studies indicate a reduction by 0.8 pK units in the intrinsic pH of some 16 carboxyl groups shown to behave abnormally upon Ca^{2+}-binding with little or no effect by Mg^{2+} ions. Alteration of the ionizability of these carboxyl groups in the regions of the Ca^{2+}-binding sites may reduce electrostatic repulsive forces by facilitated cation-binding and could contribute to a more stable, compact structure as evidenced by the greater helix content in regions III and IV (Iida, 1979).

The binding of Ca^{2+} to the Ca^{2+}-specific sites appears to produce more subtle structural perturbations (Nagy and Gergely, 1979; Levine et al., 1978). Circular dichroic (CD) analysis of observed far UV changes were interpreted as a possible addition of a peptide unit or two to one or more of the pre-existing helical segments in the NH_2-terminal half of the TnC. However this possible "tightening" of the helix coil is not considered the major influence of Ca^{2+}-binding on structure in regions I and II (Nagy and Gergely, 1979). Fluorescence (Johnson et al., 1979) and proton nuclear magnetic resonance ('H-NMR) studies (Levine et al., 1978) have resolved spectral changes associated with Ca^{2+}-binding to low-affinity sites on TnC which point to a modification of the tertiary fold of the molecule and indicate environmental changes around several hydrophobic side chains that contribute to a particular structure in the absence of Ca^{2+}.

Specific tertiary alterations in the regions of the Ca^{2+}-specific sites upon Ca^{2+} binding have been described utilizing 'H-NMR data of tryptic and thrombin TnC fragments (Evans et al., 1980). Broadening of several resonances corresponding to the hydrophobic side chains of phenylalanine, leucine and isoleucine suggest local changes in their environment attributable to tertiary-fold changes; in particular, Ca^{2+}-binding involved a marked increase in the interatomic distance between the interacting phenylalanine and methyl groups on a relatively fast time scale. On the basis of spectral data and the lack of helical changes induced by Ca^{2+}-binding to sites I and II, Evans et al. (1980) proposed that the tertiary field of TnC includes hydrophobic contacts between two pairs of phenylalanine side chains, each pair comprising one of the phenylalanine residues in the A-helix of region I containing phe 19, 23 and 26 and one of phe 72 or 75 located in the D helix of site II. These pairs contribute strongly towards stabilization of the secondary and tertiary structure in regions I and II. In response to

Ca^{2+}-binding, the two interacting helical stretches of TnC may bind or slide relative to each other while maintaining weaker hydrophic contact, thus destabilizing the helical segments of regions I and II. The magnitude of this destabilization is much less in intact TnC as compared to peptide fragments which suggests that regions III and IV may somehow constrain the motion produced by Ca^{2+}-binding at the low-affinity sites. Portions of regions III and IV may also undergo some structural change as a result of their interaction with regions I and II (Evans et al., 1980). Two lines of evidence indicate that a segment on the NH_2 terminal side of site III is integral to the interaction of TnC with TnI (Leavis et al., 1978; Weeks and Perry, 1978). Furthermore, the susceptibility of this region to Ca^{2+}-binding at sites I and/or II may transmit structural changes from these regions via III and IV to TnI and thereby be implicated in the release of TnI inhibition of actin-myosin interaction (Evans et al., 1980).

Interaction of TnC with Subunits TnI and TnT

The activating effects of Ca^{2+} involve a change in TnC that is somehow transmitted to TnI which in turn influences its association with F-actin-tropomyosin (Perry, 1980). A number of spectroscopic techniques such as fluoresence intensity changes of attached fluorophores and spin-label mobility have demonstrated the Ca^{2+}-dependent nature of TnC-TnI and TnC-TnT interactions (Mannherz and Goody, 1976). TnI and TnT are both insoluble at low ionic strength but can be solubolized by interacting with TnC at 0.1 M KCl (Hitchcock, 1975).

With regard to the TnC-TnI interaction, dissociation of the TnC-TnI complex does not occur upon chelation of Ca^{2+} ions by EGTA (ethylene bis-[oxyethylene nitriol] tetracetic acid) in contrast to its Ca^{2+}-dependent formation, nor does cation-removal completely reverse the neutralization of Mg^{2+}-stimulated ATPase inhibition imparted by TnC binding to TnI (Weeks and Perry, 1978). Ca^{2+} ions and TnI appear to exert reciprocal effects on TnC where TnC-TnI association increases the Ca^{2+}-affinity constants (Potter and Gergely, 1975) which, in turn, is reflected by an apparent strengthening of the TnC-TnI interaction (Weeks and Perry, 1978) thus suggesting a cooperativity in binding of Ca^{2+} and TnI to TnC (Perry, 1980).

Syska et al. (1976) clarified some details of TnI interaction with TnC by demonstrating the binding of two proteolytic fragments of TnI to TnC sepharose affinity columns. Additional experiments revealed that a region close to Ca^{2+}-binding site III formed complexes with TnI in the presence of Ca^{2+} (Leavis et al., 1978; Weeks and Perry, 1978). The site of interaction was identified as residues 89-100 on TnC which contains a number of negatively charged sidechains (Glu 92, 93, 94 and 97) (Leavis et al., 1978). The CNBr (cyanogen bromide) peptide CB9 of TnC (residues 84-135) containing the single cysteine residue (cys 98) was shown to possess all the properties of whole TnC. It was 50% as effective on a molar basis in neutralizing the inhibition of Mg^{2+}-stimulated ATPase activity by TnI. This peptide also blocked the phosphorylation of TnI by 3':5'-cyclic AMP-dependent protein kinase at TnI ser 117 providing further evidence for the interaction of TnC residues 89-100 with TnI residues 96-116 (Weeks and Perry, 1978). The Ca^{2+}-requirements for TnC-TnI interaction may pertain to the cation-induced folding of residues 92-102 of TnC into an α-helix (E helix) which results in the alignment of several glutamate sidechains along one side of the helix (Nagy and Gergely, 1979; Perry, 1980).

A second region on TnC involving residues 46-55 also appears to interact with the N-terminal region of TnI (Leavis et al., 1978; Weeks and Perry, 1978). Weeks and Perry (1978) demonstrated the binding of CNBr peptide CN8 (residues 48-88) to TnI and suggested that when Ca^{2+} is bound some constraint is imposed on the peptide so that the groups at the adjacent interacting regions are brought into a more favourable conformation conducive to interaction with TnI. That this interaction depends on Ca^{2+}-binding to the Ca^{2+}-specific sites has been suggested on the basis of 'H-NMR studies on TnC and its fragments (Perry, 1980). In support of this, Evans et al. (1980) suggested that the displacement of helical segments in regions I and II associated with Ca^{2+}-binding may influence residues 49-55, which form the surface-accessible loop between the regions and also the potential second TnI binding site.

The Ca^{2+}-dependent interaction of TnC with TnT appears to involve the finding of the NH_2-terminal half of the TnC to residues 159-229 of TnT (Pearlstone and Smillie, 1980). The most likely region on TnC may be located in region I (Grabarek et al., 1982). While the relative importance of all the various interactions remains controversial, Ebashi (1980) considers the TnC-TnT interaction non-essential while that of TnC-TnI essential.

b) Troponin I

The troponin-inhibiting unit (TnI) is a glubular constituent of the troponin complex so-named when identified as the protein which specifically-inhibits the Mg^{2+}-stimulated ATPase of actomyosin (Hartshorne and Meullar, 1968). Structural differences found to exist among TnI from different species and muscle types may modify the regulatory activity of this protein (Wilkinson and Grand, 1978), though all TnI species share the following properties: 1) specific inhibition of actomyosin

Mg^{2+}-stimulated ATPase, 2) neutralization of the inhibition upon interaction with TnC in a Ca^{2+}-dependent manner, and 3) substrate for phosphorylation reactions at specific sites by either a phosphorylase kinase or protein kinase (Syska et al., 1976).

Rabbit fast skeletal muscle TnI is composed of 179 amino acids with a calculated molecular weight of 20,864 d (Wilkinson and Grand, 1975), whereas rabbit cardiac TnI is distinguished by an additional N-terminal sequence of 26 amino acids which, together with other smaller additions, yields a sequence length of 215 residues (Wilkinson and Grand, 1978). On SDS polyacrylamide gel electrophoresis bovine cardiac TnI migrates with a mobility corresponding to a molecular weight of 28,000 d, while that of rabbit fast skeletal TnI corresponded to 23,000 d (Cole and Perry, 1975). Rabbit cardiac and fast skeletal TnI share only 103 of 179 identical residues (Talbot and Hodges, 1981). Charged and hydrophobic residues are evenly distributed along the highly basic TnI molecule with the exception of two hydrophilic regions where basic charges are clustered between residues 102 to 135 and 5 to 27 of skeletal TnI and with homologous regions in cardiac TnI (Wilkinson and Grand, 1975; 1978). A comparative study of rabbit fast and slow skeletal TnI, chicken fast skeletal TnI and rabbit cardiac TnI revealed that approximately 40% of the amino acids were charged imparting a net positive charge of 8 or 9 for the fast skeletal TnI, and 14 and 18 for the cardiac and slow skeletal counterparts respectively. The greater net positive charge of the latter two was attributed to a combination of increased basic lysine and arginine residues and a decrease in the acidic aspartate and glutamate residues (Wilkinson and Grand, 1978). Based on the calculated percent difference between pairs of proteins the fast muscle TnI's were more closely related than to either the cardiac or slow muscle TnI. Twice as many mutations were observed in the N-terminal half by comparison with some indication that the N-terminal halves of cardiac and slow skeletal TnI bore more similarities than any other combination. The primary structure also included two regions with concentrations of proline residues in the areas of residues 50 and 110 of skeletal TnI and homologously residues 77 and 137 of cardiac TnI. Proline residues are typically associated with bends in the molecular structure dividing the TnI molecule into roughly three equal sections. This characteristic, together with the highly polar nature of the protein suggests a fairly open structure for the TnI protein (Wilkinson and Grand, 1978). Predictions of secondary structure have given values of 45% α-helic and 10% β-sheet (Wilkinson and Grand, 1978) which are at variance with values of 20% and 29% for α-helix and β-sheet respectively obtained from circular dichroic and optical rotatory dispersion methods (Wu and Yang, 1976). Less is known regarding the tertiary and quaternary configuration of TnI.

Troponin I from skeletal and cardiac muscles can be phosphorylated at specific sites by 3':5'-cyclic AMP-dependent protein kinase and phosphorylase kinase (Perry, 1979). The major sites in fast skeletal muscle are serine-117 by the protein kinase and threonine-11 by the phosphorylase kinase, both of which are blocked upon interaction with TnC (Moir et al., 1974; Cole and Perry, 1975). Cardiac muscle TnI is phosphorylated by 3':5'-cyclic AMP-dependent protein kinase at its unique serine 20 with a much-increased rate relative to skeletal TnI, while phosphorylation at serine 146 of cardiac occurs at a more comparable rate to skeletal TnI (Cole and Perry, 1975; Moir et al., 1980). Unlike the skeletal TnI, interaction of cardiac TnI with TnC does not prohibit phosphorylation activity and may underlie functional differences between actin-myosin interaction of skeletal and cardiac systems. Table A-1 provides a summary of myofibril phosphorylation sites.

Interaction with Actin

Although TnI's inhibitory capacity was realized by Hartshorne and Meullar (1968), early binding studies utilizing TnI in combination with other thin-filament proteins were hampered by the insolubility of TnI at physiological ionic strength (0.10-0.12 M KCl) (Mannherz and Goody, 1976). Maximum inhibition by TnI on acto-heavy meromyosin free of other regulatory proteins was achieved when one molecule of TnI was present per monomer of actin although lesser inhibition occurred at the physiological TnI:actin ratio of 1:7. In the presence of tropomyosin the inhibitory efficiency of one TnI molecule was extended over 3 or 4 actin monomers implying some form of cooperativity between the actin monomers and tropomyosin (Perry et al., 1972). Tropomyosin alone inhibits actin-myosin interaction by 50-60% at 0.10 M KCl or 5 mM $MgCl_2$ which is further increased to 100% by the addition of TnI. Under similar conditions, TnI appears to increase the affinity of tropomyosin for F-actin, thereby decreasing the actin-cofactor activity further (Eaton et al., 1975).

Combined evidence from enzymatic, electrophortic and affinity chromatrographic studies on cyanogen bromide (CNBr) peptides of skeletal TnI have identified two regions of its primary structure involved in interactions with actin and troponin C. Specifically, the peptide CN4 comprising residues 96-116 was shown to interact with actin and troponin C while the N-terminal peptide CF_2 (residues 1-47) complexed with TnC (Syska et al., 1976). With regard to TnI-actin interaction, the

TABLE A-1

SITES OF PHOSPHORYLATION OF THE MYOFIBRILLAR PROTEINS
(Source: S.V. Perry, 1979)

Deleted due to copyright laws.

inhibition of acto-myosin interaction achieved by peptide CN4 is qualitatively similar to that of whole TnI in terms of the strong potentiation by tropomyosin and neutralization by TnC. This interaction is not regarded as simply electrostatic due to the basic nature of CN4 and whole TnI, since an actin-sepharose column eluted other basic proteins while retaining only TnI (Syska et al., 1976). No inhibitory activity by the shorter peptide of residues 104-112 was demonstrated in the Syska et al. (1976) study, however Talbot and Hodges (1981) clearly illustrated that the minimum inhibitory region comprised residues 105-114. Shorter peptide inhibited in a tropomyosin-enhanced manner and with an activity of 45% of whole TnI on a molar basis. In a similar study, a synthesized peptide of residues 101-115 exhibited inhibitory activity corresponding to approximately 30% of whole TnI on a molar basis (Nozaki et al., 1980).

The importance of amino acid exchanges in the inhibitory region was examined using five peptide analogs; one peptide represented the inhibitory region of chicken and rabbit fast and rabbit slow skeletal TnI, another the inhibitory region of cardiac TnI, with the remaining three peptides being combinations thereof (Talbot and Hodges, 1981). The changing of proline 110 to threonine, mimicking one skeletal-cardiac difference, did not appear to alter inhibitory activity. However, the replacement of skeletal arginine 113 with leucine and the accompanying loss of a charged sidechain resulted in decreased inhibition such as is observed in comparisons between skeletal and cardiac TnI effectiveness. In both skeletal and cardiac actomyosin systems, rabbit fast muscle TnI has been shown to be a more effective inhibitor than cardiac Tn-I perhaps explainable by structural differences in the inhibitory region (Talbot and Hodges, 1981). Due to the highly conserved nature of actin, a change in TnI sequence of sufficient magnitude could plausibly modify function. Aside from arginine 113 in the skeletal TnI, lysine 105 and valine 114 are also essential to inhibitory activity while other basic residues such as lysine 107, arginines 108 and 112 may also be important (Talbot and Hodges, 1981). Cardiac TnI shares several of these amino acids in homologous positions.

Proton magnetic resonance (PMR) studies conducted on rabbit fast skeletal TnI have verified the sites of interaction with actin and TnC and have as well provided information on specific residue involvement (Grand et al., 1982; Dalgarno et al., 1982). Titration of the peptide CN4 with actin resulted in spectral perturbations arising from the charged side chains of arginine residues, while titration of whole TnI with F-actin in a molar ratio of 1:7 influenced arginine as well as leucine and/or valine, lysine and glutamate residues, all of which predominate in the C-terminal segment of the inhibitory region. On the basis of the PMR data, it was suggested that different regions of the CN4 inhibitory peptide (residues 96-116) were involved in interactions with actin and TnC; the C-terminal portion containing the actin-binding site with the N-terminal portion providing the TnC site (Grand et al., 1982).

It has been proposed that the complimentary binding site for TnI on actin in a skeletal muscle system may entail a "hydrophobic pocket" to accommodate the valine residue and at least two acidic residues for ionic interactions with lysine 105 and arginine 113 or more if other basic residues prove essential. However no such complimentary sequence has thus far been defined in actin structure (Talbot and Hodges, 1981).

Interaction with Troponin C

The solubolization of TnI at low ionic strength and 10 μM Ca^{2+} by the addition of TnC in equimolar amounts provided some evidence for the specific interaction between the two proteins (Mannherz and Goody, 1976). The Ca^{2+}-dependent nature of this interaction was further demonstrated using polyacrylamide-urea gels on which TnI and TnC formed a strong complex even in the presence of 6 M urea, provided that Ca^{2+} was present in the system (Head and Perry, 1974). In the presence of 0.10 M EGTA the troponin subunits could be dissociated utilizing similar techniques (Perry et al., 1972). Neutralization of the inhibitory effect of TnI on actomyosin ATPase is the prime function of TnI-TnC interaction although neutralization itself appears insensitive to Ca^{2+} concentration. There is also some evidence for the interaction of the intact troponin molecule with TnC in the absence of Ca^{2+} as TnC was observed to neutralize inhibition of the Mg^{2+}-stimulated ATPase of desensitized actomyosin even in the presence of EGTA (Perry et al., 1972; Grand et al., 1982).

Electrophoretic and affinity chromatographic data have elucidated two binding sites on TnI for TnC. Selective cleavage procedures of TnI using cyanogen bromide (CNBr) yielded three interactive peptides, CF2 (residues 1-47), CN5 (residues 1-21) and CN4 (residues 96-116) (Syska et al., 1976). Additional evidence of two binding sites in skeletal muscle has accrued from phosphorylation studies where the phosphorylation of threonine-11 and serine-117 were both blocked when TnI complexed with equimolar amounts of TnC suggesting the association of TnC with TnI in these regions (Cole and Perry, 1975; Moir et al., 1974). Peptide CN4 is more strongly bound to TnC than is the shorter N-terminal peptide CN5 (residues 1-21). However, the larger N-terminal peptide, CF2 (residues 1-47), is more strongly bound to TnC than is CN5 indicating that additional residues outside the first 21 may be involved. While the most likely second binding site appears to lie between residues 9 and 21, electrostatic interactions between the largely acidic TnC and basic TnI may also contribute to complex formation (Syska et al., 1976).

Two proton magnetic resonance (PMR) studies have further clarified the specific association of the CN5 and CN4 peptides with TnC (Grand et al., 1982; Dalgarno et al., 1982). The technique of PMR permits the monitoring of signals emitted from particular residues which serve as intrinsic probes for study of sidechain environments at different points along each peptide sequence. Complex formation is thus reflected as alterations in segmental mobility and chemical shifts and/or relaxation times of the various signals (Grand et al., 1982). The addition of CN5 to a solution of TnC resulted in decreased intensities of signals corresponding to the y-CH_3 group of threonine-11, the β-CH_3 group of alanines 9 and 12, the σ-CH_3 group of isoleucine 10 and the σ-CH_2 groups of arginines 6, 8, 13 and 14 (Grand et al., 1982). Addition of spin-labelled TnC at cys 98 to a CN5 solution produced paramagnetic broadening at similar residues but also included histidine 16, the technique being sensitive at a spatial proximity of ≤ 15 Å to the label (Dalgarno et al., 1982). These results implicate primarily the central portion of the peptide CN5 in the surface matching between the TnI N-terminal region and TnC. Probe experiments also suggested a U-shaped disposition of complexed CN5 about the bound probe at TnC cys 98. The addition of 5 μM EGTA to the solution with subsequent loss of bound Ca^{2+} resulted in the loss of spectral perturbations associated with the reactive residues and indicated the necessity of

Ca^{2+} for the interaction of the CN5 with TnC (Dalgarno et al., 1982; Grand et al., 1982).

Titration of TnC into a solution of peptide CN4 in the presence of Ca^{2+} induced a broadening of the composite signal derived from the aromatic sidechains of phenylalanines 100 and 106, as well as a broadening of signals from the ε-CH_2 groups of lysines 98, 105 and 107 and the σ-CH_3 groups of leucines 99, 102, and 111. In contrast to CN5-TnC interaction, little involvement of arginine resudies was observed for CN4 thereby implicating the N-terminal portion of CN4 in surface contact with TnC. Although PMR is a powerful tool for fixing the sites of protein-protein interactions, the resolutions used in the two cited studies do not permit distinction between resonances of groups of same amino acids at different but proximal points of a peptide sequence and therefore exact borders of binding sites cannot yet be determined (Grand et al., 1982). Dissociation of the TnC-CN4 complex was apparent upon removal of Ca^{2+} by EGTA; this complex also appeared weakened at ionic strengths exceeding 0.10 M KCl (Grand et al., 1982). Signals emitted from titration of both CN4 and CN5 into solution with TnC or spin-labelled TnC in the presence of Ca^{2+} indicated that both peptides complexed non-competitively with TnC and at distinct sites (Grand et al., 1982; Dalgarno et al., 1982). Histidine-16 and phenylalanires-100 and -106 appear to be disposed at distinct sites with similar radial distance from the spin-label at cys 98 of TnC (Dalgarno et al., 1982).

Differential Ca^{2+} sensitivity of the binding of the two peptides to TnC has been shown where reduction in Ca^{2+} concentration effected by EGTA led first to the loss of paramagnetic broadening of signals associated with CN5 (histidine 16) with no alteration in those of

CN4 (Dalgarno et al., 1982). Studies employing the intact TnI molecule indicated that segmental mobility of one- or two-threonine y-CH_3 groups in the CN5 region decreased upon complex formation with TnC, this being an effect dependent upon Ca^{2+}-binding to the lower affinity, 'regulatory' sites in the N-terminal half of TnC (Evans et al., 1980; Grand et al., 1982). It therefore seems evident that Ca^{2+}-binding to low-affinity sites regulates TnC-TnI interaction at the N-terminal site on TnI. Furthermore, a functional role for the TnI N-terminal site could involve modulation of the TnI-TnC interaction that occurs on the TnI segment interacting with actin (Dalgarno et al., 1982). Stronger binding of TnC to the TnI could conceivably displace or decrease the binding of actin to TnI and thus form part of the mechanism by which interaction of Ca^{2+} with troponin controls the actin-activated ATPase (Grand et al., 1982).

Interaction with TnT

Until recently direct binding between TnI and TnT (tropomyosin-binding subunit) has been discounted (Mannherz and Goody, 1976; Horwitz et al., 1979). Both TnI and TnT are insoluble at physiological strength and tend to aggregate even at high salt concentrations which has encumbered attempts to define the relationship of the two proteins (Horwitz et al., 1979). One earlier study succeeded in demonstrating cross-linking between TnI and TnT through reactions involving dimethyl imidoester reagents and also reported the proteins to lie within 0.6 nm of each other (Hitchcock, 1975). The techniques of near ultraviolet circular dichroism (CD), which detect aromatic amino acid and cystinyl residues and reflect the tertiary structure of proteins, and gel filtration chromatography have been successfully applied to possible TnI-TnT complex formation. A strong negative CD spectra arose from a TnI-TnT mixture which was most intense at 1:1 molar ratio while gel filtration demonstrated that a stable 1:1 complex was formed between TnT and TnI which was independent of salt concentration from 0.15 to 0.50 MKCl (Horwitz et al., 1979). The interaction of TnI and TnT was observed to possess a well-defined stoichiometry and be calcium-independent as well. Far and near ultraviolet CD measurements on a 1:1 mixture of cardiac TnI and TnT also provided definite evidence for specific interaction of the two proteins perhaps involving environmental alterations of some tyrosine residues (Hincke et al., 1979). In addition, specific sites on TnT have been defined which show interaction with whole TnI (Pearlstone and Smillie, 1980).

The importance of sulfhydryl (-SH) groups in both TnC and TnI as well as in other myofibril proteins has been demonstrated in several studies (Leavis et al., 1978; Perry, 1979; McLachlan and Stewart, 1976; Ramirez et al., 1979; Chong and Hodges, 1982). These sulfhydryl groups are suspected in the proximity of the sites of protein-protein interactions. In order to identify the sites of interactions in the presence and absence of Ca^{2+}, heterobifunctional cross-linking reagents have been specifically attached to -SH groups on TnI, TnC and in whole reconstituted troponin in a 1:1 molar ratio (Chong and Hodges, 1982). The necessity of maintaining the -SH groups in the reduced state to obtain maximal interaction between TnI and TnT (Hincke et al., 1979) and to confer Ca^{2+} sensitivity in the actomyosin ATPase (Horwitz et al., 1979) has been shown. Study of the accessibility of the -SH groups of cysteines 48, 64 and 133 of TnI utilizing the cross-linking reagent iodoacetamide revealed that cysteines 48 and 64 are protected in native troponin independent of calcium and also in the TnI-TnT complex. Protection appears to be afforded by steric-blocking of one or both cysteines by

TnT and/or by the TnC, when Ca^{2+} is present, or by burial in TnI due to conformational changes induced by TnT and/or TnC. Inaccessibility in the absence of Ca^{2+} appears attributable solely to TnT. Cysteine 133 of TnI is exposed both in native troponin and in the TnI-TnT complex independent of Ca^{2+}. It therefore appears that all three cysteine residues on TnI, as well as the one on TnC, are insensitive to Ca^{2+}-induced conformational changes as regards their accessibility to reaction with iodoacetamide (Chong and Hodges, 1982). However, evidence for Ca^{2+} sensitivity of the quaternary structure of reconstituted troponin as well as for fluorescence differences in the micro-environment of cysteine 133 with and without Ca^{2+} suggest that the -SH groups may influence protein interactions in a Ca^{2+}-sensitive manner (Sutoh, 1980; Chong and Hodges, 1982).

c) Troponin-T

The tropomyosin-binding subunit (TnT) of troponin interacts with tropomyosin and the two other troponin subunits establishing communication between all thin filament proteins with full Ca^{2+} sensitivity. Troponin-T positions one troponin complex every 385 Å along the thin filaments (Flicker et al., 1982; Mannherz and Goody, 1976).

Troponin-T in rabbit skeletal muscle is a single polypeptide chain of 259 amino acids with a calculated molecular weight of 30,503 d (Pearlstone et al., 1976). On SDS polyacrylamide gel electrophoresis rabbit skeletal TnT and chicken breast TnT demonstrated migration mobilities corresponding to molecular weights of 37,000 and 45,000 d respectively (Mannherz and Goody, 1976). This difference between calculated and SDS gel molecular weights reflects TnT's anamolous elution properties due to an asymmetric shape and self-association properties

(Horwitz et al., 1979). The TnT protein is highly basic with an overall net charge of +9 at neutral pH. Over 50% of all residues are charged with the NH_2-terminal residues (1-39) being predominantly acidic and those of the COOH terminal (221-259) predominantly basic. Of a total of 61 aspartate and glutamate residues, 18 are located in the NH_2-terminal segment which is devoid of basic residues excepting 4 histidines. Similarly, the basic COOH-terminal segment possesses 13 of the total 64 arginine and lysine residues with only 2 acidic residues. There is a tendency for the remainder of the charged residues to be grouped into clusters of up to 7 residues in length. There is almost a complete absence of extensive stretches of non-polar residues and therefore indicates lack of a non-polar core. Since the relative proportion of non-polar core to surface residues is small molecular asymmetry is implied. Predictions of secondary structure indicate approximately 37% α-helix overall, 80% of which is found between residues 80-102 and 122-146. Four sections of β-sheet are situated largely in the COOH-terminal half of TnT (Pearlstone et al., 1976).

On prolonged incubation with a phosphorylase kinase a total of 3 mol phosphate per mol TnT can be incorporated into the protein indicating 3 sites of phosphorylation. These sites have been identified as the NH_2-terminal serine-1 (Site I), serines 149 or 150 (Site II) and serines 156 or 157 (Site III) (Moir et al., 1977). Only serine-1 is significantly phosphorylated at a level of 60-70% of maximum prior to two weeks phosphorylation (Moir et al., 1977). Comparison of adjacent amino acid sequences to sites of phosphorylation on TnT, TnI and phosphorylase a by phosphorylase kinase suggest some homogeneity in structure and function. Hydrophobic residues are located on the immediate NH_2-terminal side and, on the COOH terminal side, a cluster of basic residues are located

3 to 5 residues removed from the serine or threonine phosphorylated (Pearlstone et al., 1976). These similarities suggest similar mechanisms and non-specific enzymes action, however there is more recent evidence to suggest that a specific troponin-T kinase may exist (Kumon and Villar-Palasi, 1979).

Interaction with Tropomyosin

The insolubility of TnT at ionic strengths below 0.30 M KCl, which initially hampered binding studies, can be overcome with addition of polyanions or TnC in the presence of Ca^{2+} (Mannherz and Goody, 1976). Indications of TnT-tropomyosin (Tm) interaction were obtained from observations of enhanced viscosity upon mixing of the two proteins, which was sensitive to ionic strength (Jackson et al., 1975). Cleavage of TnT by cyanogen bromide (CNBr) at its five methionine residues produced six CNBr fragments which were characterized and their binding capacities to Tm assessed through chromatographic and electrophoretic techniques. Two peptides, CNB_1 (residues 1-151) and CNB_2 (residues 71-151) were found to interact with Tm each in an equimolar manner. The region of CN_2 was designated the primary binding region with enhancement of binding contributed by the NH_2-terminal portion (Jackson et al., 1975). Electron microscope studies have revealed that the majority of the troponin complex is associated with the COOH-terminal third of Tm via TnT and that residues 197-217 as well as cys 190 of Tm participate (McLachlan and Stewart, 1976). However recent evidence suggests that the highly helical CB_2 fragment of skeletal TnT is involved in the binding of the troponin complex close to or at the COOH-terminal end of Tm (residues 258-284) (Pato et al., 1981). Further evidence that the CB_1 and CB_2 fragments bind to the latter part of Tm rather than to the cys 190 region arises from studies where quantitative removal of Tm residues 274-284 by carboxypeptidase digestion markedly reduced Tm's interaction with the TnT fragments. In addition, iodination of tyrosine residues 161 and 167 of Tm was significantly reduced in the presence of CB_1 (Mak and Smillie, 1981). This evidence strongly suggests that one site of TnT-Tm interaction occurs between residues 258-284 of tropomyosin and residues 71-151 of TnT. Interaction of TnT at the COOH-terminal of Tm may explain the enhanced head-to-tail polymerization of Tm observed with TnT association (Pato et al., 1981). This interaction is not Ca^{2+}-sensitive and therefore constitutes the Ca^{2+}-independent link between the troponin complex and the other thin filaments (Mak and Smillie, 1981; Pearlstone and Smillie, 1981; 1982).

In order to reconcile the apparently contradictory electron microscope and biochemical binding data, a second binding site on TnT for Tm was proposed and identified (Pearlstone and Smillie, 1981). A chymotryptic digest of TnT yielding peptides T_1 (residues 1-158) and T_2 (residues 159-259) revealed that both peptides bound Tm (Ohtsuki, 1979). The T_1 peptide contains the first established helical site on TnT for Tm. Investigation of the T_2 peptide disclosed that residues 228-259 were essential for binding. Another fragment, B_2 (residues 206-258) was also shown to bind Tm. The order of binding strength for TnT fragments was assessed as $T_2 > B_2 > T_3$ (residues 228-259) and the secondary binding site on TnT reported to lie between residues 197-259 (Pearlstone and Smillie, 1981). This region also interacts with TnC. It was proposed that this TnT site constitutes the electron-microscope-detectable association between the troponin complex and the COOH-terminal third of Tm close to cys 190 (Mak and Smillie, 1981; Pearlstone and Smillie, 1981).

Clarification of the nature of TnT-Tm interaction has been presented from the study of Ca^{2+}-sensitive interactions of the T_1 and T_2 fragments with Tm in the presence of TnC and TnI (Pearlstone and Smillie, 1982). The two peptides were observed to bind at distinct and separate sites on Tm. The binding of T_1 to Tm was Ca^{2+}-insensitive. However, the binding of T_2 to Tm was disrupted by the addition of TnC in the presence of Ca^{2+} leading to the formation of a T_2-TnC complex. No disruption occurred in a Ca^{2+}-free condition. Similar results were obtained using the TnCI complex with Ca^{2+}. Thus, the troponin complex appears to bind to the thin filament through a Ca^{2+}-insensitive site and also a Ca^{2+}-sensitive site involving residues 197-259 of TnT and the cys 190 region in the COOH-terminal third of Tm (Pearlstone and Smillie, 1982; Mak and Smillie, 1981).

Interaction with TnC

The solubolization of TnT by TnC in the presence of Ca^{2+} as well as the formation of a TnT-TnC complex under physiological conditions provided early evidence of their interaction (Mannherz and Goody, 1976; Jackson et al., 1975; Perry, 1979). In addition, the inhibition of phosphorylation at sites II and III of TnT in the presence of TnC supports TnT-TnC interaction (Moir et al., 1977). Cyanogen bromide and enzymic fragments of rabbit skeletal TnT tested for binding on a TnC Sepharose-affinity column revealed that COOH-terminal fragments P_2 (residues 159-209) and B_2 (residues 206-258) bound most strongly to the TnC column. The interaction between the very acidic TnC and the basic TnT region of residues 159-259, occurrs in a Ca^{2+}-sensitive manner (Pearlstone and Smillie, 1978).

Interaction with TnI

The interaction of TnT with TnI in both skeletal and cardiac muscle has successfully been demonstrated after difficulties associated with the solubility properties of the two proteins hampered earlier studies (Horwitz et al., 1979; Hinke et al., 1979). In order to define the binding site(s) of TnI on TnT, various fragments of rabbit skeletal TnT derived from chemical or proteolytic cleavage were mixed with whole TnI and applied to a Sephadex column. The binding sites for TnI were found to span two distinct regions of the TnT molecule. The highly acidic NH_2-terminal portion of TnT (residues 1-70) with a net charge of -15½ provides one link to the basic TnI protein. This acidic region of TnT is peculiar to the remainder of the predominantly basic molecule. A second unique site of interaction with TnI encompasses residues 152-209 of TnT. This region has a net charge of +7½ and therefore its association with TnI would not likely be due to non-specific ionic interaction. Thus, it appears that the TnC site spanning the COOH-terminal region of TnT is either overlapping or adjacent to the second TnI site since fragment P_2 (residues 159-209) binds both TnC and TnI (Pearlstone and Smillie, 1980).

Two different types of periodicities have been noted in TnT amino acid distribution. Fast Fourier transform analysis disclosed that in the CB_2 fragment (residues 71-151) only (more specifically between residues 80-146) distinct periodicities of 8.75 and 8.68 occurred in the linear arrangement of acidic and basic residues respectively (Parry, 1981). This highly helical region of periodicity could extend over 100 Å. Parry (1981) searched for significance of this periodicity by similarly analysing tropomyosin structure. The only region in Tm that satisfied the complimentary criteria and 8.7 periodicity occurred between residues 121-173 which has not yet been shown to interact with TnT and consequently lends no support to a relationship between TnT-CB_2 periodicity and Tm association (Parry, 1981). The periodicity in the

CB_2 fragment contains some irregularities and a low α-helix potential between residues 109-121 (Parry, 1981). The periodicity may therefore be significant in that it permits the antiparallel structural arrangement of CB_2 where the two helical regions (80-102 and 122-146) are folded back on each and stabilized by ionic interactions between complimentary acidic and basic residues (Mak and Smillie, 1981; Parry, 1981). This confirmation is particularly important and plausible in view of the well-defined domains for binding of TnC (159-259) and TnI (residues 1-70 and 159-209). The fact that TnI involves two distinct binding sites on either side of the helical, periodic CB_2 region implies that the latter region may be folded such that the NH_2- and COOH-terminal regions of TnT are juxtaposed permitting simultaneous interactions with both TnI and TnC (Mak and Smillie, 1981; Pearlstone and Smillie, 1980).

A second region of periodicity occurs in the strong heptapeptide repeat of hydrophobic residues between residues 197 and 250 of TnT (Parry, 1981; Mak and Smillie, 1981). This repeat is similar to that seen in Tm and is typical of a coiled-coil structure. On this basis, it has been proposed that one type of TnT-Tm interaction may involve formation of a triple-stranded coiled-coil structure between the cys 190 region of Tm and residues 197-250 of TnT (Mak and Smillie, 1981; Pato et al., 1981).

Summary of TnT Interactions

The following diagram illustrates the sites of the thin filament protein interactions with TnT. When known, the corresponding site on the interacting protein is given in parentheses (Figure A-1).

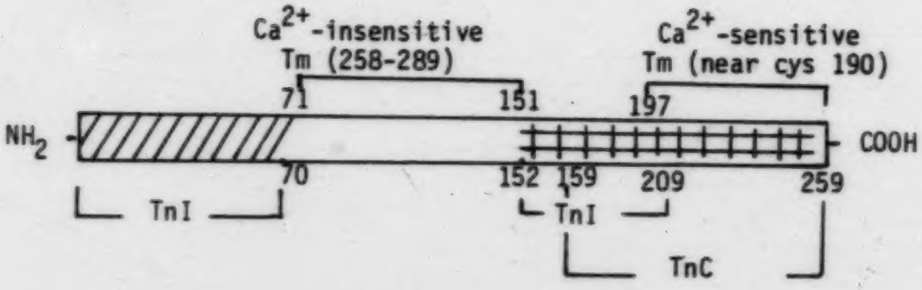

Figure A-1. Schematic diagram of troponin T associations

Troponin-T and Troponin Complex Organization

The TnT molecule, in summary, appears to be a very specialized, functionally-efficient protein composed of distinct domains which are responsible for binding to TnC, TnI and tropomyosin (Pearlstone and Smillie, 1980). Moreover, this protein is no longer thought to be globular in shape as are the other troponin components. Rather, it is seen as an asymmetric, elongated molecule capable of spanning much of tropomyosin's length (Horwitz et al.; Flicker et al., 1982).

New information on the shape of TnT, the other troponin components, tropomyosin and regarding organization of these proteins has been derived from electron microscope studies, some ulitizing rotary shadowed molecules (Ohtsuki, 1979; Flicker et al., 1982). Early electron microscope studies placed the troponin binding site near the region of cys 190 of tropomyosin (Stewart, 1975). Immunoelectron microscopy has disclosed unexpectedly large distances between antibody staining regions on the thin filaments from which the conclusion was made that TnT is elongated while TnC and TnI bind together at one end of the complex (Ohtsuki, 1979). Further study revealed a rod-like and globular domain with a length of 285 ± 40 Å reported for the entire length, while the tail or rod portion occupies 160 ± 35 Å. A length of 195 ± 25 Å was reported for isolated TnT which corresponds to the tail region indicating that the 100 Å diameter of the globular portion is most likely composed of TnC and TnI in a somewhat flattened complex (Flicker et al., 1982). Shadowed images have further clarified the location of troponin along the tropomyosin molecule. The globular portion of troponin binds 100 to 200 Å from one end of the molecule (Flicker et al., 1982) which is consistent with the earlier studies (Stewart, 1975).

Electron microscope and biochemical evidence suggest a somewhat revised description of the arrangement of troponin on tropomyosin. Cross-linking studies show that cys 190 of tropomyosin may interact with all three troponin subunits (Lamkin and Tao, 1982) while interaction of TnT and TnI (Pearlstone and Smillie, 1980; Hitchcock et al., 1981) and TnT and Tm at two sites (Pearlstone and Smillie, 1981; Mak and Smillie, 1981) have recently been confirmed. Therefore, the regulatory complex is pictured with TnC, TnI and part of TnT binding near cys 190 of tropomyosin about one-third of the way from its COOH-terminus, with the remainder of TnT extending along tropomyosin's length towards the COOH-terminus. The unusual shape of the troponin complex and, in particular the elongated TnT tail, suggests that extensive interactions between tropomyosin and TnT may be involved in the dynamics of the switching process for actin-myosin interaction (Flicker et al., 1982).

II. FUNCTION

Excitation-Contraction Coupling

Muscle contraction results from series of linked excitatory and contractile processes. Extracellular calcium acts as the essential coupler of the excitation-contraction processes and in both skeletal and cardiac muscle the use of Ca^{2+}-sensitive probes such as aqueorin has shown that an increase in myoplasmic-free Ca^{2+} concentration precedes contraction (Fabiato and Fabiato, 1979; Adams and Schwartz, 1980). The distribution of Ca^{2+} in the cell is compartmentalized and participates at several stages in the excitation-contraction pathway which begins with excitation of the sarcolemma and ends with Ca^{2+}-binding to troponin.

With regard to activation, depolarization of the sarcolemma and transverse tubule system initiates Ca^{2+} fluxes which are dependent on an extracellular source of Ca^{2+}. Increased permeability to Ca^{2+} during the plateau phase of the cardiac action potential provides the major route for transsarcolemmal Ca^{2+} influx. In skeletal muscle, this process occurs much more rapidly as is mirrored by the spiked action potential. The nature of the link between excitation and contraction processes remains obscure (Fabiato and Fabiato, 1979).

In adult mammalian myocardium, the sarcoplasmic reticulum (SR) appears to participate in the beat-to-beat contractile activation and relaxation processes as opposed to frog ventricular muscle. One proposed mechanism of excitation-contraction coupling involves Ca^{2+}-induced Ca^{2+}-release and is based on studies utilizing force-development of skinned cardiac muscle as an indication of Ca^{2+} availability (Fabiato and Fabiato, 1979). In this mechanism, there is no direct activation of the myofibrils by the net influx of Ca^{2+} across the sarcolemma; however this influx somehow triggers an even greater release of Ca^{2+} from the SR achieving the free myoplasmic Ca^{2+} concentration of greater than $10^{-5}M$ required for activation. The tight correlation between Ca^{2+} conductance and tension observed during voltage clamp experiments where a graded response of Ca^{2+} release occurred in response to graded levels of free Ca^{2+} supports the Ca^{2+}-induced Ca^{2+}-release hypothesis (Fabiato and Fabiato, 1979; Endo, 1982). An alternate hypothesis suggests that Ca^{2+} release may be induced by the depolarization of the sarcolemma and transverse tubules and bears more similarities to the proposed mechanisms for skeletal muscle.

In skeletal muscle beat-to-beat regulation is indenpendent of extracellular Ca^{2+}. Two coupling mechanisms have been postulated. The electrical coupling mechanism suggests that Ca^{2+} release is controlled by depolarization of the surface membranes and transverse tubules. Voltage-dependent charge movement within the junctional SR region results in the release of Ca^{2+} from the lateral cisternae. The mechanical coupling mechanism was proposed in the absence of evidence for tight control of activation by changes in surface membrane potentials. Mechanical links corresponding to foot processes at SR-transverse tubule junctions could couple the charges from the T tubules to sites that are then inserted into channels in the SR membrane opened by depolarization. The coupling mechanism may involve components of all the proposed mechanisms (Fabiato and Fabiato, 1979; Adams and Schwartz, 1980).

Relaxation in both mammalian skeletal and cardiac muscle relies predominantly on sequestration of Ca^{2+} which results in dissociation of Ca^{2+} from troponin. Ca^{2+} is taken up into the longitudinal tubules of the functional SR followed by transport of Ca^{2+} to the region of junctional SR for subsequent release. Ca^{2+} efflux occurs against a large concentration gradient and requires an energy-dependent active mechanism. In the SR this mechanism involves a Ca^{2+}-transport ATPase which acts as a Ca^{2+} pump and appears to account for almost all Ca^{2+} extrusion. In the sarcolemma, Na^{+}-Ca^{2+} exchange as well as a Ca^{2+}-ATPase pump may participate in Ca^{2+} efflux (Fabiato and Fabiato, 1979; Adams and Schwartz, 1980).

Myofibril Contractile Process

H.E. Huxley's (1969) sliding filament model of muscle contraction has emerged as the accepted physiological, qualitative explanation of muscle contraction. The sliding filament model asserts that in the Ca^{2+}-activated contraction, the myofilaments remain at a relatively constant length while overlapping arrays of filaments slide past each other with actin interdigitating further with the myosin filaments as the muscle shortens. The underlying mechanism producing this movement resides in the attachment of the myosin cross-bridges to actin, the cross-bridges acting as mechanical agents through which force is transmitted. The production of force is a result of a specifically determined set of structural changes in the protein complex and is associated with the cyclic ATP hydrolysis. The force-generating mechanism is able to operate over a range of actin-myosin distances due to flexible couplings at either end of the projecting cross-bridge, one of which corresponds to the coiled-coil hinge region of the S-2 region of the cross-bridge (Taylor, 1979; Huxley, 1969). When the contractile force is generated the linkage is under tension not compression, thereby facilitating the transmission of force. An important feature of this model is the preferential attachment of the myosin head to actin at an angle θ, a rotation through the angle $\theta_2 - \theta_1$, giving rise to filament sliding, followed by a detachment at angle θ_2. The cross-bridge movement could occur by: 1) outward movement of the stem, 2) a change in tilt, 3) a change in azimuth, or 4) by a combination of some or all three (Taylor, 1979).

According to Huxley (1969) the tension beyond rest is proportional to filament overlap and is therefore proportional to the number of cross-bridges potentially capable of interacting with the thin filament. Following interaction, it is suggested that cross-bridges return to the relaxed orientation although X-ray diffraction data make such a conclusion difficult. Upon activation, the well-ordered diffraction patterns of relaxed muscle are lost presenting difficulties in determining the behavior of the cross-bridges. However, since the rotational relaxation time of the S-1 head occurs in the μsec range, while tension development and return to relaxed diffraction patterns is measured in the millisecond range, reorientation of the cross-bridge before rebinding to actin is considered plausible on a timescale basis (Taylor, 1979). The direct relationship between the number of cross-bridges in the vicinity of the thin filament and tension generated, as well as the presumed reorientation of each cross-bridge to the relaxed state before rebinding postulated in Huxley's model (1969), may not completely represent the contractile mechanism. Pertaining to the first point, Podolsky et al., (1976) demonstrated, using low-angle X-ray diffraction on frog sartorius muscle, that the decrease in force exerted in an actively shortening muscle, in contrast to isometric contraction, appears due primarily to the influence of motion on the configuration rather than the number of cross-bridges. Further to this finding, Matsubura et al. (1980) studied cardiac muscle tension production during systole and diastole and proposed that tension is roughly proportional to the product of the number of heads in the vicinity of the thin filament and the degree of thin filament activation. With regard to the second point, X-ray diffraction data on skeletal muscle suggests that some cross-bridges may not return to the orientation of the relaxed state upon detachment which could in fact correspond to a 'potentiated' state of the myosin heads enhancing further interactions (Taylor, 1979). In cardiac muscle, it has been shown by X-ray diffraction, that a considerable number of myosin heads remain near the thin filament during the diastolic phase without contributing to any tension generation. An increase in this number corresponded to increased rates of systolic tension development as well as peak systolic tension which led the researchers to propose that the myosin heads may be primed to produce tension upon activation more readily than heads which have reached the complete relaxed orientation (Matsubura et al., 1980). Thus it appears that while the number of cross-bridges available for attachment to actin is important in tension generation, the state of these cross-bridges, potentiated or primed versus relaxed, may also be a contributing factor.

Studies on the mechanical properties of muscle contraction based on H.E. Huxley's cross-bridge model have contributed further information on force generation and cross-bridge behavior. Huxley and Simmons (1971) employed stepwise length changes to study the time course of resulting tension changes during contraction. They observed a rapid, nonlinear recovery of tension to preceding levels, the rate constant of which depended on the direction and magnitude of the length step. In order to explain these observations, Huxley and Simmons (1971) postulated that the cross-bridge contained an independent elastic element which permits the cross-bridge attachment site to undergo substantial displacement (length changes) relative to the filament to which it belongs. In addition, their model subdivides the force generating event into distinct stages with one for attachment and another for stretching the cross-bridges where the stretching step occurs more rapidly than attachment.

It was also necessary to assume that each cross-bridge has at least three stable positions with progressively lower potential energies through which rotation occurs. The elasticity component allows transfer from one position to another without displacing the entire filament (Huxley and Simmons, 1971).

The important contribution of Huxley's and Simmons' work (1971) towards developing a cross-bridge model lies in the concept of an elastic element in the cross-bridge permitting the cross-bridge states to exist over a wide range of axial positions (50-100 Å) capable of generating force. This concept has been further developed and modified by Eisenberg and Hill (1978) who postulate that the changes in the elastic properties of the cross-bridge are directly related to the biochemical states of the cross-bridge. In the Huxley-Simmons' model the time-dependent transitions occurring elsewhere in the cross-bridge (i.e., rotation of the myosin head on actin) indirectly affect the elastic properties of the cross-bridge which leads to the prediction of a restricted choice of rate constants. In Eisenberg's and Hill's model (1978) the rate constants are less restrictive, the key point being that the elastic properties do not determine the rate constants between the cross-bridge states. The biochemical kinetics of the actomyosin ATPase located on the cross-bridge will be presented before further brief explanation of Eisenberg's and Hill's cross-bridge model since it combines both mechanical and biochemical models of contraction.

Biochemical Models

Kinetic models of actomyosin ATPase have been derived from steady-state and pre-steady-state (transient) kinetic data obtained using the soluble proteolytic fragments on myosin, the two-headed HMM fragment and the single-headed S-1 fragment (Taylor, 1979; Eisenberg and Greene, 1980). Transient measurements constitute the main source of information regarding intermediate actomyosin states. It is recognized that the biochemical data is somewhat limited due to the necessity of studying mechanisms in homogenous solutions that may differ from the concentrations and lattice constraints of the intracellular system (Taylor, 1979). However, important features of the in vivo state are retained in solution including marked actin-activation of the myosin ATPase at low ionic strength and the integrity of ATP and actin binding sites on the parent myosin molecule (Lowey et al., 1969; Eisenberg and Greene, 1980). The methods employed in actomyosin studies have included intrinsic fluorescence of tryptophan, absorption at 190 nm, fluorescence or fluorescence polarization of bound labels, stopped-flow fluorescence, light scattering, proton release or absorption coupled to a pH indicator, conductance and direct measurements of ADP and phosphate formation (Taylor, 1979).

In the generation of actomyosin models, each biochemical state should correspond to a definite molecular structure which is separated from other possible conformations by an activation energy barrier. These states may be defined by a preferred orientation angle (θ) of the cross-bridge. Intermediate steps in ATP hydrolysis by actomyosin can then be examined over a wide range of pH conditions, ionic strength and temperature and the most likely pathway determined (Taylor, 1979). Most kinetic models are expansions upon a simple, 4-state model consisting of myosin, 2 substrate states and 1 product state where $M + T \xrightleftharpoons{1} MT_1 \xrightleftharpoons{2} MT_2$

$\xrightleftharpoons{3}$ MD $\xrightleftharpoons{4}$ M + D (M = myosin; T = ATP; D = ADP) (Taylor, 1979). In this basic mechanism, step 1 is a rapid equilibrium, step 2 is essentially irreversible while step 4 is slow compared to step 3.

1. Model of Eisenberg and Moos

Figure A-2 presents the kinetic scheme for ATP hydrolysis by myosin and actomyosin. The early model of Eisenberg and Moos (1968) attempted to account for a major result of steady-state kinetic data, the linearity of a double reciprocal plot of ATPase activity versus actin concentration. The V_{max} intercept of this plot revealed maximum actin-activated ATPase activity to be 100-200 times greater than that for HMM or S-1 alone while the K_{app} intercept (actin concentration required for half maximal ATPase) was much weaker than the binding constant of S-1 or HMM to actin in the absence of ATP (Adelstein and Eisenberg, 1980). The Eisenberg and Moos model proposed that each myosin head contained a separate ATP and actin binding site in which ATP binding greatly weakened actin binding ($K_3 < K_2$) while binding of actin to myosin activated the myosin ATPase ($K_6 < K_5$). The myosin head or cross-bridge was attached to actin at a 45° angle following ATP hydrolysis while with bound ATP in the relaxed state the angle of attachment was 90°. Transition between these two states resulted in work production (Eisenberg and Greene, 1980). Drawbacks of this model included: 1) inability to account for the transient kinetic behaviour of myosin alone including a rapid ATP hydrolysis with slower release of ADP + Pi, 2) it did not attempt to integrate physiological properties of the cross-bridge model (Huxley and Simmons, 1971) with the biochemical data, namely the elasticity component of the cross-bridge, and 3) it provided no mechanism for the binding of ATP to detach myosin from

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Figure A-2. Kinectic Models of the Myosin and Actomyosin ATPase Cycles (Adelstein and Eisenberg, 1980)

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Figure A-2 (Continued)

actin during each cycle of ATP hydrolysis (Adelstein and Eisenberg, 1980; Eisenberg and Greene, 1980).

2. Model of Lymn and Taylor (Figure A-2)

Transient kinetic data revealed a significant step in ATP hydrolysis, the initial Pi burst, the rate of which was considerably faster than the V_{max} of actomyosin ATPase (Lymn and Taylor, 1971). The magnitude of the Pi burst was given as 0.7-0.8 mol per site. These investigators also discovered that at low actin concentrations the rate of acto-HMM dissociation by ATP was much slower than the initial Pi burst. In addition a major assumption of their model was that ATP hydrolysis occurs only after M·ATP detaches from actin. Following the initial Pi burst the myosin-product complex reassociates with actin which causes more rapid release of the products ($A \cdot M \cdot D \cdot Pi \rightarrow A \cdot M$) than for myosin alone. Since the rate of the Pi burst far exceeded that of the ATPase V_{max}, Lymn and Taylor proposed that there was a rate-limiting step involved in product release after reassociation of the M·ADP·Pi complex with actin. The Lymn and Taylor model also provided a mechanism for the cyclic attachment and detachment of the myosin cross-bridge and actin. Prior to ATP binding there is strong interaction between myosin and actin at 45°. Following dissociation and ATP hydrolysis, the myosin head and actin reassociate at a 90° angle. Release of ADP·Pi involves a rotation of the myosin head from 90° to 45° thereby producing force. A major problem with this model was its exclusion of the existence of the states $A \cdot M \cdot T \rightleftarrows A \cdot M \cdot D \cdot Pi$ while other kinetic studies utilizing higher actin concentrations were able to account for the existence of these states (Adelstein and Eisenberg, 1980).

3. Refractory State Model (Figure A-2)

The refractory state model of Eisenberg and coworkers retained several main features of the Lymn and Taylor model but also attempted to explain two apparent discrepancies unaccounted for in their model. Firstly, the suggestion that the rate-limiting step in the ATPase cycle is product release after myosin reattaches to actin could not be reconciled with the short time of cross-bridge association with actin in a rapidly contracting muscle. Secondly, the Lymn and Taylor model predicted that most or all of the S-1 heads should be complexed with actin when the ATPase approached maximal activity. However, other evidence was contradictory and showed that at 5°C and high actin concentration a large number of cross-bridges were in fact dissociated (Chock et al., 1976; Mulhern and Eisenberg, 1976). Therefore, Eisenberg and colleagues proposed the refractory state model where an additional step involving transition from a refractory to nonrefractory state followed the initial Pi burst and could only occur with S-1 detached from actin. This new step became the rate-limiting step in the cycle and was used to explain the dissociation of S-1 from actin at low temperatures even when the ATPase approached V_{max} (Adelstein and Eisenberg, 1980).

Pre-steady-state kinetic data also supported the refractory state model demonstrating cyclic dissociation and reassociation of the acto-S-1 complex where ATP was present in stoicheometric amounts to acto-S-1 (5°C). Under these conditions dissociation of acto-S-1 was rapid ($A{\cdot}M{\cdot}T \longrightarrow M{\cdot}T$) while reassociation ($M{\cdot}D{\cdot}Pi_R \longrightarrow M{\cdot}D{\cdot}Pi_N \longrightarrow A{\cdot}M{\cdot}D{\cdot}Pi_N$) following ATP hydrolysis had a much slower rate constant equal to the steady-state ATPase rate (Chock et al., 1976). Therefore this data also pointed to a rate-limiting step in the transition from refractory to nonrefractory; the refractory period apparently pertained to the inability of $M \cdot D \cdot Pi_R$ to rebind to actin.

A limitation of this kinetic model lay in its assumption that S-1 rapidly dissociates from actin when all the ATP is bound leading to the prediction that at V_{max} all of the S-1 would be in the state $M \cdot D \cdot Pi_R$ and therefore dissociated from actin. However, data at 0°C showed that even though the ATPase rate approached maximum a small amount of S-1 and actin remained complexed (Mulhern and Eisenberg, 1976) while at 25°C a large amount of binding could be observed between S-1 and actin close to V_{max} (Wagner and Weeds, 1979).

Regarding a physiological cross-bridge model the refractory state model appeared to provide a biochemical explanation for two observations by A.F. Huxley (Eisenberg and Greene, 1980). First, Huxley suggested that the rate constant for cross-bridge attachment to actin is quite slow and important in determining the curvilinear force-velocity curve and in explaining the levelling off of the ATP turnover rate at high velocity. Thus, slow transition from refractory to nonrefractory state would determine the rate of S-1 rebinding to actin. Secondly, in the refractory state model product release is quite rapid and appeared consistent with the short time interval of cross-bridge-actin association in rapidly shortening muscle. Initially, this model also seemed compatible with the proposal by Huxley and Simmon (1971) that rapid rotation of S-1 from 90° to 45° occurred through a series of intermediate states upon cross-bridge attachment to actin (Eisenberg and Greene, 1980).

4. Modified Refractory State Model (Figure A-2)

The studies by Stein et al. (1979) tested several major aspects of the Lymn and Taylor and the refractory models and resulted in a revised actomyosin ATPase mechanism. The high degree of binding observed between S-1 and actin close to the ATPase V_{max} could be attributed to either a rate-limiting step in product release ($A \cdot M \cdot P \cdot Pi \rightarrow A \cdot M$) as in Lymn and Taylor's model or it could be the result of incomplete dissociation of the acto-S-1 complex by ATP at high actin concentrations ($A \cdot M \cdot T \rightleftarrows M \cdot T$) (Adelstein and Eisenberg, 1980). Stein et al. (1979) found kinetic evidence to support the latter explanation and furthermore showed that this binding occurred instanteously remaining at a constant level during ATP hydrolysis. The amount of binding depended on actin concentration hyperbolically where the amount of actin required for half-maximal binding was four times greater than that required for half-maximal ATPase activity. It was proposed that a rapid equilibrium occurred between M·T and A·M·T which was shifted towards A·M·T at high actin concentrations and towards M·T at lower concentrations. Reversibility of dissociation of A·M by ATP was also demonstrated in support of Stein et al.'s model (Sleep and Hutton, 1978).

An important result of Stein et al.'s (1979) research was the finding that ATP hydrolysis in the initial Pi burst could occur without dissociation of the acto-S-1 complex and that the step $A \cdot M \cdot T \rightarrow A \cdot M \cdot D \cdot Pi$ could occur at about the same rate as $M \cdot T \rightarrow M \cdot D \cdot Pi$, thereby refuting a fundamental point in the Lymn and Taylor mechanism. Also, it was demonstrated that a key assumption of the refractory state model was incorrect. The refractory state $M \cdot D \cdot Pi_R$, like M·T, was shown to bind weakly to actin (rejected by earlier models) while transition from refractory to nonrefractory ($M \cdot D \cdot Pi_R \rightarrow M \cdot D \cdot Pi_N$) occurred at the same rate whether complexed to actin or not (Stein et al., 1979; Eisenberg and Greene, 1980). Therefore the refractory state is not refractory to binding to actin but rather some other kinetic aspect of the transition appears to be rate-limiting (Adelstein and Eisenberg, 1980).

The major points of the modified refractory state model which is presently the currently accepted model are 1) that all of the myosin states with bound phosphate are in rapid equilibrium with their respective actin bound states, 2) that ATP hydrolysis can occur without dissociation of actomyosin (direct hydrolysis), 3) that a large free energy drop occurs upon ATP binding to actomyosin, and 4) that a slow rate-limiting transition follows the initial Pi burst (Eisenberg and Greene, 1980).

Kinetic Steps in the Myosin and Actomyosin Cycles

Individual kinetic steps in the ATPase mechanism in the absence and presence of actin will be briefly reviewed according to the Modified Refractory State Model of Stein et al. (1979).

Myosin Cycle: The upper horizontal pathway of Figure A-2 refers to the ATPase mechanism in the absence of actin. The following points have been established or proposed for this mechanism:

1. The binding constant (K_1) of ATP to myosin is approximately 10^{10} or 10^{11} M^{-1} therefore a very large free energy change occurs in this step. This step probably involves formation of a collision-intermediate followed by a conformational change as in a two-step reaction (Bagshaw and Trentham, 1974; Taylor, 1979; Adelstein and Eisenberg, 1980).
2. The second order rate constant for ATP binding (K_1) is about 10^6 $M^{-1} \cdot sec^{-1}$. The binding and forward rate of ATP-induced conformational change are very fast while the reversal appears smaller by several orders of magnitude therefore the binding of ATP is essentially irreversible (Chock et al., 1979; Taylor, 1979).

3. The initial Pi burst (K_5) has an equilibrium constant range of 1-10 indicating that almost no free energy change accompanies hydrolysis (Bagshaw and Trentham, 1974). The forward rate constant increases with increasing pH and ionic strength, its values ranging from 20 to 200 sec^{-1} (Chock et al., 1979; Adelstein and Eisenberg, 1980). The hydrolysis step then is more than 200 times faster than the overall ATP hydrolysis rate and almost 5 times faster than the V_{max} for acto-S-1 ATPase (Stein et al., 1979; Adelstein and Eisenberg, 1980).
4. Considerable controversy still surrounds the existence of a conformational change in the transition from refractory to non-refractory states (K_7) as distinct from Pi release. However the postulated rate of this transition is equal to that of its corresponding actin complex and to the V_{max} of acto-S-1 ATPase (Adelstein and Eisenberg, 1980; Taylor, 1979).
5. The product release steps (K_9 and K_{11}) probably both involve two-step reactions where the conformational change associated with Pi release (K_9) is thought to be very slow in the absence of actin and is rate-limiting above 5°C in the myosin cycle (Bagshaw and Trentham, 1974). The value of K_9 is approximately 1 M^{-1} to 10 M^{-1} and at an in vivo Pi concentration of about 5 mM a significant free energy change will accompany Pi release. However, unlike ATP binding, this step may be reversible (Cardon and Boyer, 2978; Bagshaw and Trentham, 1974; Adelstein and Eisenberg, 1980).
6. The release of ADP (K_{11}) is the rate-limiting step of the myosin cycle at temperatures below 5°C and the binding constant is about 10^5 M^{-1} (Bagshaw and Trentham, 1974).

Actomyosin Cycle: The actomyosin cycle represents the complete ATPase mechanism and has two components: 1) association and dissociation of actin with myosin through the various steps of ATP hydrolysis and product release represented by vertical arrows (K_2, K_3, K_{13}, K_{14}, K_{15}) in Figure A-2 and 2) transition of the A·M·ATP state through the kinetic steps as represented by the lower horizontal pathway (K_4, K_6, K_8, K_{10}, K_{12}) in Figure A-2. The vertical, then horizontal, relationships will briefly be discussed.

1. The binding constant of S-1 to actin (K_2) has been reported as between 10^7 to 10^8 M^{-1}. The binding constant is strengthened with decreasing ionic strength, is weakened 30-fold by ADP and by 3000-fold by ATP or ADP+P_i (Stein et al., 1979; Greene and Eisenberg, 1980). Therefore M·D and M will bind ATP more strongly compared to M·T, M·D·Pi_R and M·D·Pi_N (Stein et al., 1979).
2. The second order rate constant for A+M $\longrightarrow$ AM is reported as 5 x 10^6 $M^{-1}\cdot sec^{-1}$, a rate expected for unimpeded diffusion-limited binding (Adelstein and Eisenberg, 1980; Taylor, 1979).
3. At 50 μM actin a rapid equilibrium between M·T, M·D·Pi_R and A·M·T, A·M·D·Pi_R respectively occurs and therefore these states probably have similar second order rate constants A+M → AM. It has been suggested that, if this equilibrium exists, the differences between the binding constants of the myosin states and actin would be attributable to differences in the rates of dissociation rather than association (Stein et al., 1979; Adelstein and Eisenberg, 1980). M·T and M·D·Pi_R would rapidly detach from actin with a rate of 10^3 sec^{-1}.
4. The association of A·M with ATP (K_4) is probably a two-step reaction as in the myosin cycle with a very rapid rate constant of at

least 10^3 sec^{-1} to compete with the rapid dissociation of A·M by ATP (Stein et al., 1979). The binding constant (K_9) is quite high at 10^7 M^{-1} and at an in vivo ATP concentration of about 3 mM a large free energy drop (9.5 Kcal) accompany ATP binding to A·M making the forward reaction irreversible (Adelstein and Eisenberg, 1980; Taylor, 1979).

5. The hydrolysis of ATP on A·M (K_{16}) is a controversial step in the model and may only occur at high actin concentrations since the binding of M·T and M·D·P_{iR} to actin are quite weak. Evidence that this step does occur lies in the lack of ATPase inhibition at high actin concentrations where the accumulation of M·T and M·D·P_{iR} would be expected if not capable of binding to actin (Stein et al., 1979) and also in studies measuring ATP hydrolysis at high actin concentrations (Sleep and Hutton, 1978). In fact, the initial P_i burst on A·M·ATP may be even faster than that on myosin alone (Stein et al., 1980).
6. Researchers now agree that a slow step follows ATP hydrolysis and can occur with S-1 attached to actin however the exact nature of this step remains arguable (Taylor, 1979; Adelstein and Eisenberg, 1980). While Lymn and Taylor (1971) supported rate-limiting product release, Stein et al. (1979) defined the slow step as a rate-limiting transition from A·M·D·P_{iR} to A·M·D·P_{iN} (K_8) which precedes P_i release and occurs at about the same rate whether attached to actin or not ($K_7 = K_8$). However, it has been postulated that this rate-limiting step may more favourably occur with S-1 detached from actin in order to explain the fourfold difference between the actin dependence of the ATPase (K_{app}) and the binding of S-1 to actin (K_{13}) (Stein et al., 1979). The difference between the K_{app} and K_{13} are essential to the refractory state theory and if P_i release were rate-limiting these

two values would necessarily be equal which has not been observed (Adelstein and Eisenberg, 1980).

7. The physical nature of the transition from refractory to nonrefractory is not yet known, although information from ^{18}O exchange experiments has led to some suggestions. The nature of the ^{18}O exchange into Pi has been suggested to occur during the ATP hydrolysis step where possible rotation of Pi in the active site would allow exchange of all the oxygen (Adelstein and Eisenberg, 1980). Since this exchange apparently decreases at high actin concentrations actin would have to interfere with the access of H_2O or P_i rotation according to Stein's actomyosin mechanism (Sleep and Boyer, 1978; Adelstein and Eisenberg, 1980). However Sleep and Boyer (1978) have suggested that ^{18}O exchange occurs after ATP cleavage in the step $M{\cdot}D{\cdot}Pi_R \rightarrow M{\cdot}D{\cdot}Pi_N$ since the rate of ^{18}O exchange is compatible with that of K_7. The effect of high actin concentration would be to increase the rate of Pi release and thus limit ^{18}O exchange (Adelstein and Eisenberg, 1980). Thus the rate-limiting transition appears to involve a bound phosphate in the metaphosphate form ($M{\cdot}D{\cdot}Pi_R$) in which addition of H_2O and ^{18}O exchange are rate-limiting (Sleep and Boyer, 1978).

8. Actin activation of the ATPase may occur through enhancement of the steps involving P_i and ADP release (K_{10} and K_{12}) in the presence of actin (Stein et al., 1979). ADP release has been measured at the very rapid rate of greater than 1000 sec^{-1} at 20°C (Adelstein and Eisenberg, 1980). The rate of P_i release is not known. However since there is also no evidence to suggest reversibility of the steps $A{\cdot}M{\cdot}D{\cdot}Pi_R \longrightarrow \longrightarrow A{\cdot}M{\cdot}D$, as occurs in the absence of actin, a large free energy drop probably accompanies Pi release from

A·M·D·Pi (Adelstein and Eisenberg, 1980).

An additional controversy surrounding the myosin and actomyosin cycles involves the behaviour and participation the two S-1 heads on each myosin. Evidence from direct measurements of ligand binding constants has variably been interpreted as a single class of site on the S-1 and HMM, as two classes and for positive cooperativity between the heads. The heads do appear to rotate freely with respect to one another and substrate presence has not yielded detectable differences in fluorescence signals from the two heads. Moreover, the heads appear capable of acting independently and even binding to different actin filaments at high actin concentrations. Therefore, the weight of evidence seems to support a single class of site where the two S-1 heads probably do not exhibit positive cooperativity at least at higher actin concentrations (Taylor, 1979; Greene, 1982).

Cross-Bridge Model of Contraction

Eisenberg and coworkers have proposed an in vivo model of cross-bridge action (Eisenberg and Hill, 1978; Eisenberg, Hill and Chen, 1980) which incorporates the biochemical scheme of Stein et al. (1979), the mechanism of sliding filaments (H.E. Huxley, 1969), the concepts of flexible hinges (H.E. Huxley, 1969) and an elastic component (A.F. Huxley and Simmons, 1971) characterizing the cross-bridge and also attempts to explain the physiological properties of muscle, chiefly the isometric transient data, described by Huxley and Simmons (1971) and Ford et al. (1977). The theoretical formalism developed by Hill has allowed the free energy changes and rate constants determined from biochemical studies to be related to force, velocity and elasticity measurements determined in physiological studies (Eisenberg and Greene, 1980). Though only theoretical, this model has practical value in providing an integrated approach to understanding muscle contraction.

The key points of this model are as follows: 1) the cross-bridge is an elastic structure, 2) all of the cross-bridge states can attach to actin over a wide range of angles, but states with bound Pi (A·M·T, $A \cdot M \cdot D \cdot Pi_R$ and $A \cdot M \cdot D \cdot Pi_N$) have preferred angles close to 90°, 3) detachment of the cross-bridge does not occur as ATP is hydrolyzed in the isometric state, 4) the velocity of muscle contraction is controlled by the rate-limiting transition from the refractory to the non-refractory state which occurs either attached or unattached to actin, 5) the cross-bridge can occur in several attached states which at certain angles are in rapid equilibrium with their respective unattached states. The concept of chemical reversibility, as in point (5), has not yet been incorporated into current models of relaxation (Taylor, 1979). It is also important to realize that all current cross-bridge models have not yet successfully been applied to the complete myofibril system having the regulatory tropomyosin and troponin proteins.

In the Eisenberg group-model the cross-bridges exist either in a 90° or 45° state; these angles were first demonstrated in the X-ray diffraction studies of Reedy et al. (1965). The cross-bridges can rotate on actin such that they do not exist exclusively at 90° or 45°. Rather, certain states prefer the 90° or 45° angle of attachment, but can rotate to higher or lower angles where they exert positive or negative force respectively. Unlike the Huxley-Simmons model (1971), the rate constants for change of state do not depend on the stretching of an elastic element in the cross-bridge.

The elastic component in this newer model permits the cross-bridge states to have variable free energy levels in muscle; however, in solution the cross-bridges exist at their minimum free energy or most stable states which has prevented direct testing of the model. According to this model, the basic free energy of the cross-bridge is a function of x, a measure of the position of the actin site relative to the cross-bridge. The distance x is directly related to the angle of the attached cross-bridge (at 90°, x = 80 Å; at 45°, x = 0). In the unattached states (M·T, $M \cdot D \cdot Pi_R$ and $M \cdot D \cdot Pi_N$) the free energies are independent of x. Eisenberg et al. (1980) developed a free energy profile which relates the chemical parameter (free energy) to physiological parameters such as cross-bridge position, x and mechanical force. The shapes of these curves are based on the elastic properties determined from the isometric transient data of Ford et al. (1977). An important feature of this model is that no work is performed in transition between states of the free energy profile; rather, work is done when the cross-bridge in an attached state moves along x while its free energy simultaneously decreases. The elasticity component, then, is manifest as a free energy gradient along which the cross-bridge moves.

Since the Eisenberg et al. (1980) model is based on the biochemical data of Stein et al. (1979), it must provide a mechanism for cyclic detachment of the cross-bridge in vivo as the muscle shortens even though in vitro ATP hydrolysis can occur with or without detachment from actin. This model must also explain how the large free energy drop associated with ATP binding to myosin is utilized in vivo. Some clues to this question have been derived from ATP analogue studies (Eisenberg and Greene, 1980). Thus, the major assumption of the model is that the conformational change associated with ATP binding to A·M not only weakens the binding of actin, but also changes the preferred angle of the attached cross-bridge from 45° back to 90°. Consequently the free energy associated with ATP binding to A·M is a function of x (sensitive to the angle of attachment to actin).

The cross-bridge in the refractory state rapidly attaches to actin in a region where it exerts positive force. The cross-bridge then makes a slow, rate-limiting transition to the non-refractory state. Following this transition, the conformational change associated with Pi release occurs rapidly at an x = 80 Å. While the conformational change induced by ATP binding changes the preferred angle of cross-bridge attachment from 45° to 90°, the conformational change accompanying Pi and ADP release returns the cross-bridge to a preferred angle of 45°. The step involved in Pi release is postulated to be quite fast and to account for the rapid isometric transient.

Analogue research by Marston et al. (1976) suggests that ADP lengthens the muscle fiber. Therefore, Eisenberg et al. (1980) propose that the release of Pi returns to cross-bridge to only 50° and that ADP release is required for full return to 45°. The ADP release may at first be slow in the more stable A·M·D state providing a mechanism whereby the cross-bridge remains attached to actin until completion of the power stroke. As the cross-bridge rotates to the x = 0 position and reaches the A·M state final steps in ADP release are very rapid. Following this release, the myosin head rebinds to actin to form the state A·M·T with a preferred angle of 90° to begin a new cycle.

Rapid equilibria between weak attached and unattached states in vitro support the possibility that in vivo, A·M·T will rapidly detach from actin when its free energy exceeds that of the unattached state M·T. This detachment, it is postulated, will only occur during shortening. In the isometric state, the cross-bridges will not detach as ATP is hydrolyzed but will oscillate between attached states and at

$x = 80$ Å, will exist mostly as A·M·D exerting positive force. The rate-limiting transition from refractory to non-refractory states will be the major determinant of the velocity of muscle contraction because it controls the ability of the cross-bridge to complete a full work cycle (Eisenberg and Greene, 1980).

Whereas the original Eisenberg model (1978) predicted that the number of attached cross-bridges will markedly decrease as shortening velocity increases explaining the decline in force with increasing velocity, the ~~revised model~~ (1980) predicts that attached cross-bridge number will not change much with greater velocities. This proposal is congruent with X-ray diffraction research on isotonically-contracting frog skeletal muscle which showed no significant changes in the number of attached cross-bridges as velocity increased (Podolsky et al., 1976).

In summary, the presence of cross-bridge elasticity in combination with the x-dependence of the rate constants and rapid equilibria between attached and unattached states may explain the observed detachment of cross-bridges from actin during shortening without this detachment being essential to hydrolysis and force production (Eisenberg and Green, 1980).

Models of Myofibril Relaxation

In vertebrate striated muscle the effect of intracellular calcium (Ca^{2+}) on the troponin-tropomyosin (Tn-Tm) complex controls contraction. At low Ca^{2+} concentrations the regulatory Tn-Tm complex inhibits actin-myosin interaction while at high Ca^{2+} concentrations this inhibition is released. The most widely accepted structural interpretation of this phenomenon is the steric blocking model put forward by Huxley (1972), Haselgrove (1972) and Parry and Squire (1973). Based on a number of X-ray diffraction and electron microscope studies, this model asserts that relaxation occurs when tropomyosin rotates away from the center of the long-pitch actin grooves, which it occupies during activation, and physically blocks the attachment of the myosin cross-bridge to actin. The position of tropomyosin is thought to be controlled by troponin. In the absence of Ca^{2+}, troponin maintains tropomyosin in its relaxed position on the outer side of the actin groove, whereas upon Ca^{2+} binding to troponin-C, the troponin allows tropomyosin to shift towards the center of the groove, presumably exposing the myosin-binding sites on the actin monomers. This mechanism has been supported by three-dimensional image reconstruction studies on decorated actin filaments (Wakabayashi et al., 1975). Due to tropomyosin's position extending along 7 F-actin monomers, it is thought to be effective over the entire actin unit and therefore cooperativity is inherent in the model (Greene and Eisenberg, 1980).

It is largely structural analyses which have lent support to the steric-blocking model although some confirmation has also been shown in biochemical studies. Bremel et al. (1972) demonstrated that at low ATP concentrations the actomyosin ATPase is not sensitive to Ca^{2+} and suggested that in this rigor state, the rigor bonds held tropomyosin in the activated position even in the absence of Ca^{2+}.

However, more recent structural and biochemical experimentation have cast doubt on the appropriateness of the original steric blocking model. There is some evidence that the Tm-Tn complex may act indirectly by inducing a conformational change in the actin monomer (Taylor, 1979; Adelstein and Eisenberg, 1980). Changes in actin pitch have been observed in the relaxation mechanism where in an actin-Tm-Tn complex the actin helix pitch is given as 13/6 but upon addition of Ca^{2+} changes to 28/13 (Taylor, 1979).

Examination of thin filament structure with attached Z lines using three-dimensional image reconstruction techniques altered previous thinking on thin filament polarity (Seymour and O'Brien, 1980). The results indicated that tropomyosin in the inhibited thin filament is located on the opposite side of the actin groove from the position required for the steric blocking model. While tropomyosin's position shifts in response to Ca^{2+}, the authors proposed regulation by conformational change or partial blocking of the myosin-binding site on actin (Seymour and O'Brien, 1980). Taylor and Amos (1981) using minimal dose electron microscopy and image reconstruction confirmed their results by suggesting that the polarity of the reconstructed images of undecorated thin filaments is opposite to that assumed in the original steric blocking model. A revised steric blocking model has thus been devised which combines the reversed thin filament polarity evidence with a change in the geometry of myosin S-1-actin interaction (Taylor and Amos, 1981). These authors report a new overall shape of myosin S-1 where more of its mass is located at the end proximal to the actin than distally. The actin binding site on S-1 has also been moved from the mid-proximal end towards the outside of the broad head. Consequently, a portion of the S-1 extends into the long-pitch helical actin groove where it appears to come into close contact with a feature identified tentatively as tropomyosin. Thus, in the Taylor and Amos model (1981) tropomyosin is still able to block the actin S-1 binding site for myosin due to the proximity of the two structures in the actin groove. The apparent position of tropomyosin in the "activated" filaments shows it on the opposite side of the actin groove well-removed from the possible site of direct actin-S-1 interaction. In the rigor complex, tropomyosin appears to be in close contact with a feature of the S-1. Taylor and Amos (1981) contend that the trapping of tropomyosin in the long-pitch helical groove upon cross-bridge binding, even in the absence of Ca^{2+}, as required in the rigor state can be explained by their model. It is also proposed that an interaction between tropomyosin and the cross-bridge may help explain the potentiation by tropomyosin observed in earlier studies (Bremel et al., 1972; Taylor and Amos, 1981).

Despite revision, the steric blocking model is unable to account for a number of experimental biochemical observations. Potentiation of the actomyosin ATPase activity has been observed at saturating levels of ATP as well if the S-1 to actin ratio is very high (Adelstein and Eisenberg, 1980). In the absence of ATP, binding of HMM or S-1 to actin strengthens the binding of tropomyosin to actin rather than interfering with it (Eaton et al., 1975). Also, in the absence of troponin, tropomyosin only partially inhibits the actomyosin ATPase (Eaton et al., 1975). Evidence from various species suggests that the tropomyosin effect may depend on the structure of the actin monomer as well as on tropomyosin position (Adelstein and Eisenberg, 1980). An important drawback to the steric blocking model is that it cannot account for possible chemical reversibility of reactions in the actomyosin ATPase cycle. Attachment and detachment of the cross-bridges may not be an all-or-none phenomenon; rapid equilibria probably occur between attached and detached states (Greene and Eisenberg, 1980). These equilibria are influenced by Ca^{2+} and therefore the binding of Ca^{2+} to troponin and the binding of cross-bridges to actin may have a synergistic effect on each other (Adelstein and Eisenberg, 1980). In addition, increased Ca^{2+} binding results in decreased rates of detachment and may therefore influence relaxation rates (Johnson et al., 1979). Collectively, these points compromise a simple steric blocking model.

Another serious consideration regarding the plausibility of an all-or-none mechanism of relaxation is that it has never been demonstrated which of the myosin states is prevented from binding to actin in the absence of Ca^{2+} (Adelstein and Eisenberg, 1980). The Tn-Tm complex may affect the binding constant of each myosin state to actin in a specific, quantitative way. Greene and Eisenberg (1980) investigated the binding of myosin S-1·ADP to regulated actin. In the absence of Ca^{2+}, S-1·ADP binding to regulated actin is strongly inhibited in a cooperative manner where at low S-1·ADP to actin ratios binding was very weak compared to unregulated actin, while at high levels of saturation the S-1·ADP binds 10^3 more strongly to regulated than to unregulated actin. This cooperative effect of S-1·ADP binding to actin in the presence of the Tn-Tm complex has been observed elsewhere (Trybus and Taylor, 1980). It has thus been proposed that the Tn-Tm action can be analyzed in terms of typical allosteric models in which, with both Ca^{2+} and EGTA, there appears to be a weak-binding and strong-binding form of the actin-Tm-Tn complex. Ca^{2+} and S-1·ADP would therefore have roles as allosteric effectors. The binding of Ca^{2+} to troponin would shift the equilibrium from the weak form to the strong form as would S-1·ADP binding to actin (Greene and Eisenberg, 1980). The lower rate of S-1·ADP binding to regulated actin in the absence of Ca^{2+} may be explained by a lower concentration of strong binding sites, perhaps one open site per 7 G-actin residues. Partial occupancy of a block of G-actin sites by S-1 or S-1·ADP would serve to stabilize the open conformation and allow greater cross-bridge binding (Trybus and Taylor, 1980). A second cooperative effect, the interaction of nearestneighbour tropomyosin molecules in an end-to-end manner, has also been invoked to help explain the cooperative transition between the relaxed and activated states (Chalovich et al., 1981).

Investigations on the nature of S-1·ADP and S-1·ADP·Pi binding to actin have revealed that the Tn-Tm complex had a relatively small effect on binding in the presence or absence of Ca^{2+}, while the actin-activated ATPase rate is inhibited 96% in the absence of Ca^{2+} (Chalovich et al., 1981). This decreased activity upon Ca^{2+} removal has been attributed primarily to a reduction in the V_{max} rather than in the apparent binding constant of S-1 to actin (Chalovich and Eisenberg, 1982). This data led to the suggestion that the Tn-Tm complex may induce relaxation by altering a kinetic step(s) in the ATPase cycle, probably Pi release from the acto-S-1·ADP·Pi complex rather than by blocking the binding of S-1·ADP·Pi or S-1·ATP to actin (Chalovich et al., 1981). This mechanism plus the weakening of the S-1·ADP binding to actin by Tn-Tm system may in turn be explained by an increase in the free energy (hence decreased stability) of the acto-S-1·ADP complex when actin is in the weak form. Thus, weak binding of S-1·ADP to the weak form of actin would be linked to an inability of the weak form to activate the S-1·ATPase (Chalovich et al., 1981).

On the basis of the cross-bridge model described by Eisenberg and Greene (1980), the Tn-Tm complex may shift the free energy curves of the 90° and 45° states in vivo thereby altering the whole free energy profile. Tn-Tm may have little effect on the binding of myosin states which attach to actin at a preferred angle of 90° (M·ATP and M·ADP·Pi) but can greatly weaken the binding states which attach to actin at a preferred angle of 45° (M·ADP and M). Thus, the 45° state would become destabilized, the rate of transition from the 90° to the 45° state would be decreased and consequently Pi release would decline (Chalovich et al., 1981).

Since native tropomyosin acts as a non-competitive inhibitor of ATP hydrolysis in the absence of Ca^{2+}, Chalovich and Eisenberg (1982) propose that tropomyosin and S-1·ATP do not compete for the same site on actin. They furthermore suggest that the Tn-Tm system acts to "sterically-block" the rotation of S-1 on actin and the accompanying release of Pi. Ca^{2+} may function either to shift the weak form of actin to the strong form or may shift tropomyosin's position on actin so that S-1 rotation is not associated with as high an activation energy and thus the rate of Pi release is increased. An additional result from this research is that a fraction of the myosin cross-bridges appear to remain attached to actin during relaxation. Although structural studies argue against this occurrence, the authors speculate that weak attachments of cross-bridges in the 90° state which exert no net force and are rapidly attaching and detaching may not be detectable on macroscopic levels. Moreover, these attachments would be flexible so as to allow the return of the myofilaments to their relaxed positions (Chalovich and Eisenberg, 1982).

In summary, it appears that there are two main bodies of thought regarding the mechanism of relaxation, that based on structural evidence and that derived from biochemical data and models. To this point, no one study has been able to present a unified perspective on this problem.

III. REGULATION

Calcium

Since the early 1960's convincing evidence has accumulated which has led to the recognition of the calcium ion as the primary regulatory factor of muscle contraction at the molecular level (Ebashi, 1980; Bremel et al., 1972; Ebashi and Endo, 1968). The major regulatory role of myoplasmic Ca^{2+}, that of activation of the myofibril ATPase, operates through the troponin-tropomyosin system (Bremel et al., 1972) where it has been shown that the degree of contractile activity corresponds to free Ca^{2+} concentration (Winegrad, 1971; Katz, 1970).

The necessity of a readily exchangeable source of Ca^{2+} for the maintenance of force production by cardiac muscle was demonstrated by Rich and Langer (1975) in skinned muscle where chelation of extracellular Ca^{2+} resulted in a rapid decline in force development by cardiac preparations while the skeletal muscle could maintain force for up to several minutes in the absence of external Ca^{2+}. The dependence of cardiac excitation-contraction coupling mechanism and of force generation on extracellular calcium has led to investigation of the potential regulatory role of this Ca^{2+} compartment on myocardial contraction. At present the exact quantity of Ca^{2+} delivered to the myofibrils from the extracellular source is unknown. The mechanism of delivery is also disputed. The triggered-release proposal of Fabiato and Fabiato (1979) which was briefly described earlier under Function holds that the extracellular Ca^{2+} current triggers the release of larger stores of Ca^{2+} from the SR into the myofilament space producing adequate activating Ca^{2+} concentrations while the alternate theory states that sufficient Ca^{2+} is transported from the extracellular space to directly activate the myofilaments (Langer, 1978).

Regardless of the coupling mechanism there is evidence to suggest a regulatory role for extracellular Ca^{2+} (Philipson and Langer, 1979; Bers and Langer, 1979). A quantitative relationship between total sarcolemmal-bound Ca^{2+} and the tension development of rabbit and rat interventricular septum and papillary muscles has been reported in these studies. Using isolated sarcolemmal vessicles Philipson and Langer (1979) documented the presence of specific binding sites for Ca^{2+} and observed that association and dissociation of Ca^{2+} from these sites modulated the kinetics of the changes in contractility accompanying the alterations in Ca^{2+} concentration. The magnitude of the developed tension at any time appears to be proportional to the fraction of occupied sarcolemmal Ca^{2+} receptors. A low-affinity receptor (K_m = 1.2 μM) is ten times more abundant than a second higher affinity receptor (K_m = 20 μM) and appears to play a regulatory role in Ca^{2+} flux (Philipson and Langer, 1979). These authors attributed the inability of some earlier studies to show a temporal relationship between low interstitial Ca^{2+} concentrations and decline in contractility to the fact that actual Ca^{2+} levels at the capillary-cell interface, shown to be morphologically close, were more regulatory where rapid binding and dissociation from the specific receptors could occur. Thus, the dissociation constant of Ca^{2+} would govern the rate of decline in contractility while the greater association constant would permit a more rapid Ca^{2+}-induced enhancement of the rate of tension development in the myofibrils. It has also been suggested that graded entry of Ca^{2+} via these receptors (and perhaps in an electroneutral transmembrane exchange for Na^+) could account for the increase in intracellular Ca^{2+} during cardiac systole, and/or could trigger a graded release of Ca^{2+} from the SR (Bers and Langer, 1979). The sarcolemmal moieties primarily responsible for the Ca^{2+}-binding properties appear to be three phospholipids which probably interact with specific membrane proteins to bind up to 80% of the total bound Ca^{2+}. Sialic acid residues appear to account for only about 13% of total bound Ca^{2+} (Philipson et al., 1980). In summary then, kinetically-identified Ca^{2+} receptors on the sarcolemmal membrane appear to participate in the regulation of myofibril activation, this mechanism being compatible with either theory of E-C coupling (Philipson and Langer, 1979).

The rise in myoplasmic Ca^{2+} levels initiates myofibril activation which proceeds in a concentration-dependent manner (Solaro et al., 1974; Winegrad, 1971). Although the dependence of contractility on Ca^{2+} was presumed to be related to the amount of Ca^{2+} bound to troponin (Bremel et al., 1972) the work of Solaro et al. (1974) determined the actual amounts of Ca^{2+} required to achieve various levels of myofibril activation in dog heart by measuring the dependence of biochemical (ATPase activity) and mechanical (tension development) correlates of activation and myofibril Ca^{2+} binding on free Ca^{2+} concentration. Myofibril Ca^{2+} binding remained low at 10^{-7}M free Ca^{2+} until 10^{-6}M free Ca^{2+} was reached at which point binding increased steeply. One-half maximal ATPase activation was achieved at 2.1×10^{-6}M free Ca^{2+} which corresponded to previous reports in rabbit cardiac muscle (Winegrad, 1971). A close correlation between the myofibril ATPase response to Ca^{2+} and that of isometric tension development where 50% of maximal tension occurred at 2.0×10^{-6}M Ca^{2+} was observed. At Ca^{2+} concentrations between 10^{-7} and 10^{-6}M the two functions differed significantly where a higher degree of ATPase activity was noted over tension. This difference was attributed to possible myosin ATPase activity (Solaro et al., 1974). Overall, both ATPase activity and tension displayed similar sigmoidal activation curves over the range of 10^{-8} to 10^{-5}M free Ca^{2+} (Solaro et al., 1974; Winegrad, 1971). A complex relationship between the activation parameters and myofibril Ca^{2+} binding was reported where maximal ATPase and tension were realized at Ca^{2+} concentrations not sufficient to saturate the myofibril sites. Thus it was suggested that only a portion of the calcium sites were required in the regulation of ATPase and tension (Solaro et al., 1974). This had previously been proposed for skeletal muscles as well (Bremel et al., 1972). In addition, a large degree of positive cooperativity in the activation of ATPase and tension was observed for both cardiac (Solaro et al., 1974) and skeletal muscle (Bremel et al., 1972) although maximum activation of cardiac muscle appears to require about one-third the amount of Ca^{2+} needed by skeletal muscle (Solaro et al., 1974).

The in vitro studies on Ca^{2+} requirements for activation have more recently been substantiated by direct measurements of in vivo Ca^{2+} levels during rest and contraction (Allen and Blinks, 1978; Marban et al., 1980) although it has been difficult to sensitively and accurately measure Ca^{2+} levels in the sub-micromolar range. Microinjection of the bioluminescent protein aequorin followed by measurement of tension development and intensity and amplitude of the light signal emitted by Ca^{2+}-aequorin interaction demonstrated that alterations in contractility are the result of changes in the Ca^{2+} transient in the myofilament space supporting the beat-to-beat regulation by Ca^{2+} in cardiac muscle (Allen and Blinks, 1978). A slow rate of rise of the Ca^{2+} transient signal furthermore provided a basis for the slower onset of activation observed in cardiac muscle. A more precise determination of free Ca^{2+} levels in heart cells was accomplished utilizing Ca^{2+}-sensitive microelectrodes (Marban et al., 1980). Agreement between the measured amounts of in vivo intracellular Ca^{2+} required to achieve various levels of tension development (Marban et al., 1980) and the calculated in vitro levels utilized by Solaro et al. (1974) was obtained. The mean resting level of Ca^{2+} measured by the microelectrodes was 2.6×10^{-7}M while under caffeine-induced contractions intracellular Ca^{2+} levels reached 1×10^{-5}M and corresponded to significant force development (Marban et al., 1980).

Examination of the site(s) of Ca^{2+} regulation of myofibril activation may be useful before further discussion of activation characteristics. Structure, binding sites and affinity constants and Ca^{2+}-induced conformational changes of TnC were dealt with in the Structure section. Before such information was available the concept of Ca^{2+}-binding to particular troponin sites as the regulatory mechanism was mentioned in the literature (Bremel et al., 1972; Solaro et al., 1974). It has been established that cardiac TnC possess two high affinity Ca^{2+}-Mg^{2+} sites and one low affinity, Ca^{2+}-specific site. Thus examination of the temporal relationships between Ca^{2+} and/or Mg^{2+} binding and dissociation from these sites and the Ca^{2+}-induced conformational changes connected with activation of the ATPase has revealed that only Ca^{2+}-binding to the Ca^{2+}-specific sites appears to regulate contractile activity (Leavis and Kraft, 1978; Johnson et al., 1979 and 1980; Evans et al., 1980). Johnson et al. (1979) reported that the Mg^{2+} off-rate of the high affinity sites was 8 sec^{-1} which would preclude activation by Ca^{2+} at these sites governed by the rate of displacement of Mg^{2+}. The timescale for Ca^{2+} binding and release at low-affinity sites was however consistent with the onset of activation (less than 50 ms.) and relaxation. Further support for Ca^{2+} regulation at the low affinity site has been provided by a fluorescent probe study of Ca^{2+} binding to Ca^{2+}-specific sites of rabbit cardiac TnC and reconstituted troponin (Johnson et al., 1980).

Holroyde et al. (1980) demonstrated that in bovine heart muscle, the activation of myofibril ATPase occurred over the same range of Ca^{2+} concentrations at which significant Ca^{2+} bound to the low-affinity Ca^{2+}-specific sites whereas the high-affinity Ca^{2+}-Mg^{2+} sites were filled before the onset of ATPase activation. An additional study followed the time-course of the percentage of maximal occupancy of each type of site with Ca^{2+} in response to various pCa (negative log of Ca^{2+} concentration) transients (Robertson et al., 1981). Steady-state saturation profiles indicated that there was Ca^{2+} movement on and off each class of site in response to each pCa transient but only the Ca^{2+} exchange rate with the Ca^{2+}-specific sites was analogous to the contraction-relaxation cycle. With regard to the Ca^{2+}-Mg^{2+} sites, a gradual but substantial increase in the mean level of Ca^{2+} occupancy of these sites occurred over repetitive exposures to the Ca^{2+} transients and it was therefore suggested that percent saturation of these sites with Ca^{2+} could possibly be a measure of the intensity and frequency of recent muscle activity (Robertson et al., 1981). Participation of the divalent metal ion-binding sites on myosin in regulation of activation has also been discounted since the pCa transients induced no significant Ca^{2+}-binding to these sites due to their high Mg^{2+} affinity although substantial Ca^{2+}-binding could occur on a slower timescale (Robertson et al., 1981).

The widely-held belief that only the Ca^{2+}-specific receptors on TnC regulate contractile activity has been challenged by Kohama (1979, 1980). Contraction was induced in cardiac muscle by relatively low concentrations of Sr ions which were too low to allow the low affinity site of TnC to bind Sr ions and therefore suggested an active role of the high affinity site in contraction (Kohama, 1979). Moreover, only one high-affinity and one-low affinity site were observed. A subsequent study exhibited superprecipitation of a myofibril system at very low Ca^{2+} concentrations (10^{-7}M) where only the high-affinity sites would bind Ca^{2+} (Kohama, 1980). Mg^{2+}-binding to the low affinity sites was also demonstrated which is at considerable variance with other reports in the literature for both skeletal and cardiac troponin (Potter and Gergely, 1975; Johnson et al., 1979; Robertson et al., 1981). The discrepancy between these two approaches to site-specific Ca^{2+} regulation has not yet been resolved.

The parameters of myocardial activation shown to be regulated by Ca^{2+} includes myofibril ATPase activity, tension development, immediate stiffness, the maximum unloaded shortening velocity (V_{max}) and possibly the cardiac length-velocity and length-tension relationships. The close relationship between ATPase and tension over the range of activating levels of Ca^{2+} in both cardiac and skeletal muscle is well-known (Winegrad, 1971; Solaro et al., 1974; Bremel et al., 1972; Herzig, 1978). The possibility that increased myocardial force is associated with an increase in the number of cross-bridges attached to actin at any one moment and that this number could be directly influenced by Ca^{2+} was investigated by Herzig and Ruegg (1977). They utilized the immediate stiffness measurement which has been suggested to be proportional to the number of elastic links formed between actin and myosin (Huxley and Simmons, 1971). With increasing Ca^{2+} concentrations, a proportional increase in immediate stiffness and myocardial tension was demonstrated and was paralleled by similar Ca^{2+} dependence of the actomyosin ATPase (Herzig and Ruegg, 1977). The observed relationship of tension, stiffness and ATPase was said to be indicative of a mechanism whereby Ca^{2+} ions increase isometric force development by recruitment of activated cross-bridged (Herzig and Ruegg, 1977 and 1980).

The velocity of tension development was also shown to increase under conditions where myoplasmic Ca^{2+} levels were increasing, however this effect was noted only in the range of Ca^{2+} concentrations at or below 10^{-6}M, while isometric tension and stiffness continued to increase with higher levels of Ca^{2+}. It was concluded that although Ca^{2+} does appear to influence the rate of X-bridge kinetics and contraction speed, further changes would not be mediated by Ca^{2+} alone (Herzig and Ruegg, 1977). Maximum unloaded shortening velocity (V_{max}) also differed from other activation parameters in terms of its sensitivity to Ca^{2+} as measured by the Ca^{2+} concentration required to achieve half-maximal activation. Herzig (1978) demonstrated that V_{max} requires a higher Ca^{2+} concentration for half-maximal activation than tension. Further investigation of the Ca^{2+} regulation of V_{max} revealed half-maximal activation occurred with 2×10^{-6}M Ca^{2+} while that of tension and stiffness was achieved at 7×10^{-7}M Ca^{2+}. Therefore it appears that V_{max} can be altered independently of tension and stiffness and that other factors besides the number of attached cross-bridges must determine V_{max} (Herzig and Ruegg, 1980). The lower Ca^{2+} sensitivity of V_{max} versus tension furthermore suggests that Ca^{2+} regulation of the two parameters may occur at different sites within the contractile system. The much greater sensitivity of tension, ATPase and stiffness to the inotropic agent cAMP as compared to V_{max} supports this view and some evidence indicates that cAMP-dependent or Ca^{2+}-dependent phosphorylation-dephosphorylation mechanisms on Tn-I and the myosin P-light chain respectively would regulate the response of the activation parameters to Ca^{2+} (Herzig and Ruegg, 1980).

Brenner (1980) developed a technique to differentiate between the immediate effects of any parameter on V_{max} and those effects indirectly brought about by shortening-induced changes in cross-bridge afterload during shortening. Studies on rabbit psoas muscle (Brenner, 1980) and on cat papillary muscles (Brenner and Jacob, 1980) demonstrated that shortening velocity decreased exponentially with progressive shortening during isotonic quick releases. The apparent Ca^{2+}-dependence of the length-velocity relationship was observed whereby variation of Ca^{2+} concentration results in a change in the slope and increasing Ca^{2+} levels are accompanied by a smaller decrease in velocity during the quick release. Rather than a direct effect of Ca^{2+} on V_{max}, it was proposed that the apparent Ca^{2+}-dependence be interpreted as the result of an alteration in cross-bridge afterload during the shortening and preceding the reading of velocity (Brenner, 1980). By specifically applying various afterloads and varying initial length it was demonstrated that the effect of an additional shortening of 0.2 μ per sarcomere on the shortening velocity could be simulated by an external afterload of about 1.8×10^{-5}N. This relationship was not influenced by myoplasmic Ca^{2+} concentration. It was concluded that, after a given change in length due to shortening, the increase in afterload per cross-bridge due to this shortening-induced afterload should be inversely proportional to the degree of activation of the contractile (due to Ca^{2+} concentration). This theory was invoked to explain the steeper slope of the length-velocity relationship at lower Ca^{2+} levels (Brenner and Jacob, 1980). Thus with greater Ca^{2+} concentrations and greater number of activated cross-bridges, the afterload corresponding to the particular length change due to shortening would be more widely distributed reducing the load per cross-bridge and thereby providing less resistance to further shortening.

The cardiac length-tension relationship appears to be directly regulated by free Ca^{2+} concentration. The inotropic influences on contractility mediated through elevated Ca^{2+} levels and the increased tension due to stretch previously considered to involve different mechanisms were suggested to operate through the same mechanism (Allen and Blinks, 1978). Tension development of frog cardiac muscle was observed to increase as filament overlap and amplitude of the aequorin-Ca^{2+} signal decreased. The authors proposed that a prime influence of stretch in cardiac myscle may be to increase the Ca^{2+} sensitivity of the system in order to maintain or enhance tension development (Allen and Blinks, 1978). An increase in Ca^{2+}-sensitivity of tension with increasing sarcomere length at less than-maximal activating Ca^{2+} levels has also been reported by Fabiato and Fabiato (1978b). At high Ca^{2+} concentrations and full activation however, increasing sarcomere length from 2.2 or 2.3 μM to 3.00 μM results in a reduction to 82% in frog heart (Fabiato and Fabiato, 1978). A direct proportionality between tension and stiffness was demonstrated in the double stretch experiments on rabbit trabecular preparations of Herzig and Ruegg (1977) who attribute the similarity in the stretch-induced and Ca^{2+}-induced tension and stiffness responses to an increased number of cross-bridges bound to actin at any one moment. Thus the stretch-induced Ca^{2+} sensitivity appears to result in a greater number of functioning cross-bridges. Increased tension at greater sarcomere lengths may be further enhanced by the reported length-dependence of Ca^{2+} release from the SR where Ca^{2+}-induced Ca^{2+} release is markedly inhibited with decreasing sarcomere length (Fabiato and Fabiato, 1975b). Thus, stretch in cardiac muscle results in greater performance at sub-maximal activation partly due to altered Ca^{2+}-regulation. This implies a mechano-chemical basis for the Starling mechanism.

Superimposed on the fundamental Ca^{2+}-activation of myocardial contractility are a variety of other mechanisms which serve to modulate the direct influence of Ca^{2+} on contractile activity and which may in fact be regulated by Ca^{2+} themselves either directly or indirectly. Regulation mechanisms exist at the levels of the sarcolemma, sarcoplasmic reticulum and within the myofibrils, which will chiefly be considered here. The parameters of the Ca^{2+}-activation curve which are subject to modification include: 1) the maximal tension which occurs at Ca^{2+} concentrations insuring full activation, 2) the Ca^{2+}-sensitivity which refers to the Ca^{2+} concentration required to attain half-maximal activation, and 3) the cooperativity with respect to Ca^{2+} which is represented by the slope of the activation curve (Rupp, 1980). Alteration of Ca^{2+} sensitivity provides a mechanism for modulating the degree of activation at a given intracellular Ca^{2+} concentration. Variation in cooperativity would influence both the time-course of tension development and the extent of activation. These three parameters may be altered independently of each other and may result in simultaneous effects of an apparently opposite nature. Thus the behavior of the contractile system is complex and difficult to predict (Rupp, 1980). Mechanisms through which one or more of the activation parameters may be modified include phosphorylation-dephosphorylation reactions and regulation by Mg^{2+}, MgATP, inorganic phosphate or pH.

Phosphorylation

The mechanism of phosphorylation and dephosphorylation of specific phosphoproteins in tissue has become recognized as an important regulator of biochemical and physiological processes (Barany and Barany, 1981). Typically a signal at the cell membrane elicits transmission via so-called second messengers which in cardiac tissue comprise an altered cyclic AMP (cAMP) or altered Ca^{2+} concentration. Intracellular receptors within the cell bind either cAMP or Ca^{2+} and exert influence either directly or indirectly often by a phosphorylation mechanism. The cAMP receptor is a protein kinase which upon activation catalyzes the phosphorylation of several proteins in muscle including sarcolemmal, sacroplasmic reticular and myofibrillar proteins as well as glycogenolytic intermediates. The Ca^{2+} receptors include TnC, which does not participate in phosphorylation reactions, and calmodulin which as a Ca^{2+}-calmodulin complex regulates several enzymes through a Ca^{2+}-calmodulin-dependent protein kinase (Kranias and Solaro, 1983; Barany and Barany, 1981). The phosphorylation reaction involves the protein kinase-catalyzed transfer of the y-phosphate of ATP to a hydroxyl group of serine, threonine or tyrosine residues. Formation of a phosphorylated protein, termed covalent modification, adds two negative charges to the protein and may be the underlying chemical basis for the physiological events regulated by phosphorylation. Dephosphorylation is catalyzed by specific protein phosphatases (Barany and Barany, 1981; Morgan et al., 1976).

Recognition that phosphorylation of myofibrillar proteins may contribute to the regulation of contractile activity first emerged when it was shown that both myosin and troponin I purified from rabbit skeletal muscle could be phosphorylated (Perrie et al., 1973; Moir et al., 1974). While other proteins such as tropomyosin and troponin T are phosphoproteins and may influence contractility, only mechanisms involving TnI and myosin will be considered in this discussion.

Troponin I:

Phosphorylation of TnI is the only phosphorylated myofibrillar protein for which a physiological significance has recently emerged. The identification of the additional 26 amino acid residues in the NH_2 terminus of cardiac troponin and specifically the location of a serine residue at position 20 provided structural evidence for the physiologically-observed phosphorylations (Grand and Wilkinson, 1978; Moir et al., 1980). TnI phosphorylation was first correlated with increased force of contraction (positive inotropy) in perfused rat hearts stimulated with isoproterenol or epinephrine (England, 1975). Moir et al. (1977 and 1980) demonstrated that treatment of perfused rabbit hearts with adrenaline increased the total covalently bound phosphate from 1.14 $mol \cdot mol^{-1}$ TnI to 1.86 $mol \cdot mol^{-1}$ TnI. Additional observations that the covalently bound phosphate was located primarily in the region of cardiac TnI consisting of residues 1 to 48, that serine 20 became radioactively labelled when ^{32}P-labelled orthophosphate was present in the perfusion medium and that upon addition of adrenalin the extent of serine 20 phosphorylation increased from 0.3-0.4 mol to about 1.0 mol phosphate·mol TnI^{-1} further substantiated serine 20 in TnI as the major receptor for adrenergically-induced, cAMP-dependent phosphorylation (Moir et al., 1980). This data supported that of earlier radioactive studies which identified serine 20 as the probable site for rapid exchange between the intracellular pool of phosphate and that bound to TnI in beating hearts (Solaro et al., 1976). Phosphorylation of serine 20 by cAMP-dependent protein kinase paralleled the increase in force development in a dose-dependent response to adrenalin which led to the proposal of a significant role for TnI phosphorylation in the inotropic response to catecholarine stimulation (Solaro et al., 1976). While not disputing the relationship, other studies have demonstrated that the peak of force development and the peak phosphorylation were not simultaneous with peak force occurring earlier and that TnI remained phosphorylated for some time after force had diminished (England, 1976; Westwood and Perry, 1981). Thus it was suggested that β-adrenergic stimulation resulting in TnI phosphorylation participates in a more tonic type of regulation since TnI remains phosphorylated beyond the inotropic effect (Stull et al., 1982; Resink and Gevers, 1982). Additionally it was observed that positive inotropy could be induced via other agents in the absence of cAMP-dependent phosphorylation of TnI indicating that this mechanism is not solely responsible for positive inotropy and may in fact occupy a regulatory role in the catecholamine-induced positive inotropic response (England, 1976; Resink and Gevers, 1982).

At the biochemical level Ray and England (1976) made the observation that TnI phosphorylation in the beef heart caused an increase in the calcium concentration required for activation of actomyosin ATPase. This finding has been replicated by numerous other studies using a variety of animal models (Wyborny and Reddy, 1978; Holroyde et al., 1979[c] and 1980; Resink et al., 1979; Mope et al., 1980; Yamamoto and Ohtsuki, 1982). Such evidence reversed the earlier proposal of Rubio et al. (1975) who had reported that phosphorylation by exogenous protein kinase of TnI in native actomyosin complexes from guinea pig hearts resulted in an increased Ca^{2+}-sensitivity of the ATPase rather than the now-accepted decreased sensitivity. The rightward shift in the pCa-ATPase relationship induced by TnI phosphorylation results in an increase in Ca^{2+} concentration required for ½ maximal activation from 0.2 μM to 1.0 μM (Holroyde et al., 1979[c]).

Agreement on the influence of TnI phosphorylation over the full range of myofibril ATPase activity has not been achieved. While some studies have shown no effect on the basal or maximal ATPase activity (Holroyde et al., 1979[c]), others have observed a more complex relationship. Bailin (1979) reported a decrease in bovine cardiac actomyosin ATPase upon TnI phosphorylation even at very low levels of Ca^{2+} as well as an altered Ca^{2+} sensitivity. At saturating levels of Ca^{2+}, cAMP-dependent protein kinase phosphorylation of TnI raised the concentration of Ca^{2+} required for maximal ATPase activity from 3×10^{-6}M to 1.0×10^{-5}M. Moreover, a 15-40% reduction of actomyosin ATPase activity was observed over a range of Ca^{2+} concentrations from 1.5×10^{-8} to 2.4×10^{-5}M (Yamamoto and Ohtsuki, 1982). Similar reductions in maximal ATPase activities with TnI phosphorylation were reported by others (Resink et al., 1979; Wybomy and Reddy, 1978). Resink and Gevers (1982) further demonstrated a reduction in the V_{max} $|Ca^{2+}|$ of the Ca^{2+}-Mg^{2+} ATPase activity of regulated actomyosin isolated from isoproterenol-perfused rat hearts. Perfusion in catecholamine-free perfusate resulted in dephosphorylation of TnI, presumably by endogenous phosphatases, which correlated with increases in the V_{max} and Ca^{2+}-sensitivities of the Ca^{2+}-Mg^{2+} ATPase. Alterations in the V_{max} and Ca^{2+}-sensitivity characteristics followed the same temporal patterns during the experimental procedures. The delay in Tn-I dephosphorylation observed upon β-agonist withdrawal has been suggested to reflect a mechanism dependent upon the relative concentrations of cAMP which mediates phosphorylation and cGMP believed to mediate phosphatase activity (England, 1976; McClellan and Winegrad, 1980; Resink and Gevers, 1982).

The mechanism for the reduced Ca^{2+}-sensitivity and ATPase activity has been suggested to arise from a modification in the Ca^{2+}-binding properties of TnC transmitted in response to TnI phosphorylation (Solaro et al., 1976; Holroyde et al., 1979[c]). Decreased myofibrillar Ca^{2+} binding over the entire range of activating Ca^{2+} levels was observed upon cAMP-dependent phosphorylation of dog cardiac myofibrils. Incubation with exogenous alkaline phosphatase subsequently reduced covalent phosphate content and increased Ca^{2+}-binding and Ca^{2+}-sensitivity of the ATPase (Holroyde et al., 1979[c]). In contrast, Stull and Buss (1977) were unable to detect any modification in Ca^{2+}-binding by phosphorylated myofibrils, however their methods and preparation may not have distinguished between high- and low-affinity sites on TnC (Robertson et al., 1982; Holroyde et al., 1979[c]). In this regard, Robertson et al. (1982) selectively followed Ca^{2+}-binding to the Ca^{2+}-specific sites on fluorescently-labelled TnC in reconstituted troponin. Upon cAMP-dependent phosphorylation 0.9 ± 0.3 mol phosphate was incorporated into troponin, 98% of which was located on TnI. This resulted in decreased Ca^{2+}-specific sites for Ca^{2+} which regulate ATPase activity (Robertson et al., 1981; Johnson et al., 1980) and also elicited a significant increase in the rate of Ca^{2+} removal from the regulatory sites (Robertson et al., 1982). Thus decreased Ca^{2+}-binding and increased Ca^{2+}-removal to and from the myofibrils contribute to the altered Ca^{2+} regulated ATPase activity with cAMP-dependent phosphorylation of TnI.

The effects of TnI phosphorylation on the Ca^{2+} sensitivity of the myofibril ATPase and on the V_{max} has led to some agreement on interpretation regarding the functional significance of this mechanism. It has been proposed by a number of researchers that TnI phosphorylation contributes to the well-known increased rate of cardiac relaxation observed in catecholamine-stimulated positive motrophy (Katz, 1980; England, 1976; Solaro et al., 1976; Holroyde et al., 1979[c]; Robertson et al., 1982; Resink and Gevers, 1982). This mechanism appears to act in concert with a cAMP-dependent phosphorylation of the protein phospholamban in the SR (Kranias and Solaro, 1983) which acts to increase the initial rate and extent of Ca^{2+} uptake from the myoplasmic compartment (Kirchenberger et al., 1974; Tada and Katz, 1982). The speed of relaxation is therefore enhanced by diminished thin filament sensitivity to Ca^{2+}, by increased rates of Ca^{2+} dissociation from troponin and by increased rate of uptake into the SR. It is suggested that the SR phospholamban is the prime determinant of catecholamine-enhanced relaxation while TnI serves as a modulator of the relaxant effect. Evidence that phosphorylation of the SR and of TnI occurs within the same time frame during the inotropic response supports a coordinated response to catecholamine-stimulation by the two organelles (Kranias and Solaro, 1983). While in agreement with the above proposal, Resink and Gevers (1982) amend that enhanced relaxation with TnI phosphorylation is further facilitated by reduced actin-actin transduction leading to decreased actin-myosin interaction and experimentally observed as a depressed V_{max} over the range of activating Ca^{2+} concentrations.

An additional proposal for the physiological function of TnI phosphorylation contends that it represents a negative feedback system during catecholamine stimulation (Perry, 1979). This phosphorylation mechanism may function to dampen or smooth out the positive inotropic response of the heart produced by the transient increase in Ca^{2+} concentration that results from β-adrenergic stimulation and thereby acts as a negative feedback control mechanism on the cardiac myofibril ATPase activity (Solaro et al., 1976; Moir et al., 1980).

Myosin P-Light Chain

All vertebrate myosins investigated so far contain one or more 18,000-20,000 dalton light chain components which undergo phosphorylation-dephosphorylation reactions and have therefore been termed P-light chains

(Frearson and Perry, 1975; Barany and Barany, 1981). The existence of highly specific protein kinases (Perrie et al., 1973; Pires et al., 1974; Frearson and Perry, 1975) and phosphatases (Morgan et al., 1976) which catalyze these reactions in muscle has given rise to the theory that this mechanism may exert some regulatory control on myosin's participation in contractility although the biochemical and physiological significance in skeletal and cardiac muscle remains obscure (Stull et al., 1982; Barany and Barany, 1981). The P-light chains also serve as the Ca^{2+} receptor of the thick filament (Bagshaw, 1977; Holroyde et al., 1979[a,b]) which undergoes conformational changes upon Ca^{2+}-binding in the range of activating levels of Ca^{2+} (Morimoto and Harrington, 1974; Malhotra et al., 1979). Myosin light chain kinase (MLCK) is activated by the binding of a Ca^{2+}-calmodulin (4:1) complex and catalyzes the incorporation of the γ phosphate from ATP into a serine residue near the NH_2 terminus of the P-light chain (Barany and Barany, 1981; Stull et al., 1982). The rate of myosin P-light chain phosphorylation is thus determined by the kinetics of Ca^{2+} release and uptake, the concentration and activity of MLCK, and the relative activity of the light chain phosphatase (Stull et al., 1982). Calmodulin is a ubiquitous protein and therefore is probably not a limiting factor (Barany and Barany, 1981). The relationship between Ca^{2+}-binding to and phosphorylation of the P-light chain possibly involves alteration of actin-myosin interaction due to modified Ca^{2+}-binding properties of the P-light chain (Kardami and Gratzer, 1982).

The covalent phosphate content of myosin P-light chains in beating hearts has been shown to turnover and change (Frearson et al., 1976). Freeze-clamp experiments on beating hearts in situ or in a Langendorff perfusion system measured the basal phosphate content to be 0.3-0.4 mol per mol P-light chain (Holroyde et al., 1979[a]). Other studies have determined amounts of 25% phosphorylation in controls (Westwood and Perry, 1981), 0.50 mol phosphate per mol light chain (Barany et al., 1983) or 50% basal phosphorylation (Jeacocke and England, 1980). While no concensus has been reached regarding normal basal phosphate content, most investigators agree that the P-light chains exist in a partially phosphorylated state.

The degree of P-light chain phosphorylation in heart has been correlated with the development of active tension. Since hearts were stopped in diastole and phosphate incorporation determined it was proposed that light chain phosphorylation may predetermine the amount of tension generated in the ensuing contraction (Kopp and Barany, 1979). Light-chain phosphorylation was also associated with an increase in the V_{max} of the myosin Ca^{2+}-ATPase activity in one study (Resink and Gevers, 1982) though not to a significant extent in another (Reddy and Wybomy, 1979). Enhanced ATPase activity was also reported at Ca^{2+} concentrations above half-maximal levels with light chain phosphorylation (Holroyde et al., 1979[a]). Greater functional importance may lie in the reported direct relationship between the extent of phosphate incorporation into the P-light chain and the enhancement of actin-activation of myosin Mg^{2+}-ATPase (Reddy and Wyborny, 1979). This phenomenon has also been observed to a significant extent in skeletal muscle (Pemrick, 1980). Westwood and Perry (1981) point out however, that since only 1 in 4 myosin heads is phosphorylated under basal conditions, phosphorylation is not a prerequisite for actin-activation but may be a regulator.

Investigation of the relationship between β-adrenergic stimulation, positive inotropic and P-light chain phosphorylation has led to some controversial results. A number of researchers failed to find little or any change in phosphate content at the peak inotropic response to adrenergic stimulation of cardiac muscle (Holroyde et al., 1979[a]; Jeacocke and England, 1980; High and Stull, 1980; Westwood and Perry, 1981). Catecholamine intervention in other studies however did demonstrate a relationship with P-light chain phosphorylation. Kopp and Barany (1979) observed that only the P-light chain phosphate content exhibited consistent changes corresponding to both positive and negative inotropic responses. Abrupt removal of β-adrenergic stimulation from perfused hearts furthermore led to the temporally-related decline in cAMP levels, declining heart rate and dephosphorylation of the P-light chain (Resink and Gevers, 1982). Some investigators therefore contend that changes in the P-light chain phosphate content are associated with parallel changes in the contractile state of the myocardium.

Since cardiac contractility is related to increased Ca^{2+} levels and since Ca^{2+}-binding to the light chains may modify contractility via an alteration in actin-myosin interaction, the influence of light chain phosphorylation on Ca^{2+}-binding by the light chains has been explored. Alexis and Gratzer (1976) indirectly measured Ca^{2+}-affinity by using intrinsic or dansyl probe fluorescence and observed an apparent reduction in light-chain Ca^{2+}-binding upon phosphorylation. Similarly, the Ca^{2+}-affinity of light chains was perturbed upon phosphorylation with an increase in the Ca^{2+} concentration required for 50% saturation of the Ca^{2+}-binding sites (Kardami and Gratzer, 1982). The Ca^{2+}-binding process was observed to operate in the physiological range, resulted in an increase in the α-helicity of the light chain and demonstrated apparent positive cooperativity between strong and weak binding sites (Kardami and Gratzer, 1982). In direct contrast to this, another study found no evidence of phosphorylation-regulation of light-chain Ca^{2+}-binding or of positive cooperativity in Ca^{2+}-binding to the light chains (Holroyde et al., 1979[b]). The existence of a second regulatory mechanism involving Ca^{2+} has received support since Lehman (1978) demonstrated that low to moderate Ca^{2+} concentrations activated actomyosin ATPase in the absence of troponin-tropomyosin regulation. Therefore Holroyde et al. (1979) suggested that Ca^{2+}-binding to the light chains and phosphorylation constitute separate and distinct regulatory mechanisms of thick filament involvement in contraction. Regulation by phosphorylation may represent a tonic response acting over several heart beats.

In summary, while the relationship between Ca^{2+}-binding to and phosphorylation of the myosin P-light chain is unclear both appear to influence contractile parameters. The physiological importance of Ca^{2+}-dependent light chain phosphorylation may reside in an alteration of cross-bridge kinetics where an increased negative charge on the cross-bridge drives it away from the myosin filament towards the actin filament upon activation. It may also serve to increase bonding with actin-bound Ca^{2+} and further increase the rate of combination of the cross-bridges with actin (Barany and Barany, 1980). However, discrepancies between existing studies need to be resolved before a concrete proposal can be developed.

MgATP

The $MgATP^{2-}$ complex is thought to serve a dual function in muscle. Firstly, it acts as the substrate in the actomyosin ATPase reaction where its hydrolysis provides the energy required by the contractile process. Secondly, it functions as a plasticizing agent by binding to the myosin cross-bridge active site which weakens or dissociates the actin-myosin interaction thereby maintaining muscle extensibility (Weber and Murray, 1973; Marston et al., 1979). The intracellular concentration of ATP as determined by ^{31}P nuclear magnetic resonance is given as 3 to 6 mM where it exists as a complex with Mg^{2+} due to an excess of Mg^{2+} (Burt et al., 1976).

Variation of MgATP concentration through the micromolar to millimolar range was first shown to influence tension generation in Ca^{2+}-free mediums. Work with skinned crayfish muscle revealed a bell-shaped tension curve as a function of MgATP (Reuben et al., 1971).and was later substantiated by studies on skinned frog skeletal muscle and skinned segments of single rat cardiac cells (Fabiato and Fabiato, 1975). At low MgATP concentrations in the micromolar range (pMgATP 7.0) fibers generated 15-20% of maximal tension which increased with increasing MgATP levels to 50% at pMgATP of 5.50. Higher MgATP concentrations (pMgATP 5.50 to 4.0) resulted in a decline in tension to 5-10% (Fabiato and Fabiato, 1975). Reuben et al. (1971) interpreted this relationship as a phenomenon of substrate inhibition where initially, in the absence of Ca^{2+}, MgATP would interact with the myosin head resulting in cross-bridge formation with actin as MgATP concentration increased. A plateau of activation appears to exist after which further increase in MgATP levels would result in a substrate inhibition of the myosin ATPase reaction. The explanation of Bremel and Weber (1972) and elaborated by Murray and Weber (1973) is slightly different. They interpret the presence of tension at low MgATP levels as a threshold-dependent formation of actin-myosin rigor complexes resulting from cooperative interaction within the actin filament (the "on-state"). Substrate saturation and ATP hydrolysis increase until the MgATP saturation of on units no longer compensates for the loss of actin-myosin units being switched off by higher MgATP, the rate of hydrolysis declines and finally reaches a minimum when all units are in the off state.

The physiological relevance of extremely low or high MgATP levels is debatable since MgATP concentrations are well-maintained in muscle (Godt, 1974). However, variations of MgATP between 30 μM and 4 mM and even up to 30 mM have demonstrated significant effects on isometric tension generation in cardiac (Best et al., 1977; Fabiato and Fabiato, 1975; Rupp, 1980), skeletal (Godt, 1974; Kerrick and Donaldson, 1975; Ferenczi et al., 1979; Poremel and Weber, 1972) and in skinned crayfish muscle (Brandt et al., 1972; Orentlicher et al., 1977). The parameters affected include: 1) maximum isometric tension, 2) the pCa-% maximum tension and pCa-ATPase relationships, and 3) the steepness or cooperativity of the pCa-tension curve.

Lack of uniformity exists regarding changes in maximal isometric tension. An inverse relationship between MgATP and maximal tension was reported in cardiac muscle where a 25% decrease in tension occurred as MgATP increased from 30 μM to 2 mM with no further change at 4 mM (all at 1 mM Mg^{2+}) (Best et al., 1977). Conversely Fabiato and Fabiato (1975) found a significant increase in maximal tension with a decrease in MgATP concentration (pMgATP 2.5 → 3.5). Similar declines in maximal tension with increases in substrate concentration from micromolar to millimolar levels have been reported in skeletal and crayfish muscle (Magid, 1974; Orentlicher et al., 1977). Ferenczi et al. (1979) demonstrated a hyperbolic relationship between maximal shortening velocity and MgATP concentration over the range of 10 μM and 10 mM. Steepness of the force-velocity relationship determined from isotonic releases was increased at the MgATP level of 5 mM versus 0.3 mM at a pCa of 4.5 (Ferenczi et al., 1979). Dissenting reports where maximal tension was independent of MgATP have also emerged (Godt, 1974; Brandt et al., 1972). The possible increase in maximal tension with decreasing MgATP (and vice versa) has been suggested to involve an increase in the number of actomyosin linkages at any one moment at low MgATP resulting in greater maximal force (Best et al., 1977).

The effect exerted by MgATP on the pCa-tension curve involves a clear substrate-induced shift to the right with increasing substrate and to the left with decreasing substrate (Orentlicher et al., 1977; Best et al., 1977). This phenomenon has been observed in cardiac cells (Best et al., 1977; Rupp, 1980; Fabiato and Fabiato, 1975) and in skeletal and crayfish muscle (Godt, 1974; Orentlicher et al., 1977; Fabiato and Fabiato, 1975; Kerrick and Donaldson, 1975). Biochemical data has suggested a similar activating effect of low MgATP on the myofibril ATPase rate where Ca^{2+}-sensitivity was reported to increase by one order of magnitude as MgATP decreased from 2 mM to 20 μM (Weber, 1970). Portzhel et al. (1969) observed that in rabbit myofibrils the Ca^{2+} requirements for activation were not affected by MgATP over a range of 0.3 to 8.9 mM whereas crab myofibrils exhibited greater Ca^{2+} requirements at higher MgATP.

Contradictory reports exist regarding the effect of MgATP on the steepness of the pCa-tension curve. The slope of the curve is described by the Hill n where a number greater than 1.0 denotes positive cooperativity, equal to 1.0 denotes independent binding and that below 1.0 signifies negative cooperativity of binding (Rupp, 1980). Best et al. (1977) reported increased steepness of the curve as MgATP was elevated to the millimolar range, this effect being far less dramatic at normally physiological Mg^{2+} concentrations (1 mM). Reducing MgATP from 4 mM to 100 μM resulted in a decrease of the Hill n from 1.9 to 1.2 without a change in Ca^{2+} sensitivity and resulted in an increased tension at low to midrange Ca^{2+} values (pCa 6.1) and decreased tension at higher values (pCa 4.7) thereby enhancing the tension ratio at lower Ca^{2+}. Between 100 μM and 30 μM Rupp (1980) further observed that the major effect involved increased Ca^{2+} sensitivity. As regards skeletal muscle, some results concur with those on cardiac (Kerrick and Donaldson, 1975; Orentlicher et al., 1977) while others failed to detect a change in the shape of the pCa-tension curve as a function of MgATP (Godt, 1974; Brandt et al., 1972). The physiological implications of altered Ca^{2+}-sensitivity and cooperativity as regards MgATP in cardiac muscle may pertain to an increase in systolic and particularly diastolic total tensions (with a decreasing amplitude of developed tension) as a result of MgATP depletion such as may occur with ischemia (Rupp, 1980).

Two schemes have been proposed to explain the effects of MgATP on Ca^{2+}-activation parameters. That of Bremel and Weber (1972) and Weber and Murray (1973) contends that at low MgATP the inhibitory capabilities of troponin are compromised by the formation of nucleotide-free actomyosin linkages (rigor complexes). This cooperativity leading to the formation of rigor bonds may also influence the affinity of Ca^{2+}-binding sites on troponin. At a critical level of rigor complexes the low affinity sites for Ca^{2+} become more like the high-affinity sites and consequently Ca^{2+}-activated tension is greater at lower Ca^{2+}. (Hence, the shift to the left of the pCa-tension curve with lower MgATP.) According to this scheme, increased cooperativity would also result at low MgATP however two studies on cardiac muscle have found the opposite (Rupp, 1980; Best et al., 1977).

The alternate scheme of Orentlicher et al. (1977) is compatible with that of Weber and Murray (1973). Since MgATP-dependent changes arise from the level of actin-myosin interaction (MgATP does not bind to the thin filament) Orentlicher (1977) presents a quantitative assessment which considers the ratio of uninhibited (activation with MgATP) to inhibited states (substrate inhibition at high MgATP) which are probably functions of the rate constants of the actomyosin cycle. In effect three states are proposed: ES_2, ES and E. ES_2 contains a double-bound nucleotide and corresponds to the off-state of Weber and Murray (1973). ES and E, the single bound nucleotide and nucleotide-free states, represent the on and potentiated states of Weber and Murray (1973). Thus, as substrate concentration increases, Ca^{2+}-activated tension changes from an active, potentiated state to lower levels of tension and finally to the completely relaxed, inhibited state (ES_2).

Magnesium

The regulatory potential of the free divalent cation magnesium (Mg^{2+}) at the myofibrillar level has become apparent from studies investigating modulation of Ca^{2+}-activation. The physiological range given for intracellular free Mg^{2+} in heart is between 10^{-4} and 10^{-3}M (Polemini and Page, 1973). Analysis in skeletal muscle by ^{31}P NMR spectroscopy provided a free Mg^{2+} measurement of 0.6 mM (Gupta and Moore, 1980). In this range Mg^{2+} has demonstrated a regulatory effect on certain contractile parameters related to Ca^{2+}-activation in both cardiac and skeletal muscle (Rupp, 1980; Fabiato and Fabiato, 1975; Kerrick and Donaldson, 1972; Solaro and Shiner, 1976).

The maximum tension of skinned rat cardiac fibers was not greatly influenced by variation of Mg^{2+} over a physiological range (1-5 mM) (Donaldson et al., 1978). However, increasing Mg^{2+} has been observed to elicit a reduction in submaximal tension generation in skinned frog skeletal muscle (Kerrick and Donaldson, 1972). The major effect of increasing Mg^{2+} appears to be the rightward shift of the pCa-% tension curve in both skeletal and cardiac muscle (Kerrick and Donaldson, 1975; Fabiato and Fabiato, 1975; Donaldson et al., 1978; Rupp, 1980). Thus small changes in intracellular Mg^{2+} appear to influence myofibril tension generation at a given Ca^{2+} concentration. Conversely, in the presence of elevated Mg^{2+}, the activating effect of Ca^{2+} is diminished at submaximal levels (Rupp, 1980). Variation of Mg^{2+} concentration between 1 and 5 mM had a negligible effect on the Hill n value (2.0) or steepness of the pCa-% tension curve in cardiac muscle, however a decrease in Mg^{2+} to 50 μM resulted in an increased Hill n value and decreased maximum tension (Donaldson et al., 1978). Unlike cardiac muscle, an increasing Mg^{2+} concentration showed a decrease in the Hill n value of skinned skeletal fibers (Kerrick and Donaldson, 1975). Rupp (1980) has proposed a physiological significance for possible reduction in Mg^{2+} in cardiac muscle due to anoxia, ischemia or other pathological states. The altered pCa-tension relationship would result in a shift of the length-tension relationship to higher tensions in systole and particularly in diastole. Thus, increased tension during the relaxation phase would increase ATP utilization and perhaps lead to an energy insufficiency. This has led to investigations of the possible role of increased Mg^{2+} in recovery from ischemia perhaps having an ATP sparing effect (Bersohn et al., 1982).

Biochemical alterations due to increased Mg^{2+} concentration involve a rightward shift of the pCa-ATPase relation where Mg^{2+} elevates the Ca^{2+} required to attain a given level of activation (Solaro and Shiner, 1976). In skeletal muscle increasing Mg^{2+} concentrations from 0.04 to 1.0 to 10 mM also resulted in a depressed myofibril ATPase activity over the entire range of Ca^{2+} (10^{-8} to 10^{-4}M) and an increased relative steepness of the pCA-ATPase curve. The responses in cardiac myofibril ATPase were different. At Ca^{2+} concentrations of 10^{-6}M of greater, increasing Mg^{2+}

from 0.04 to 1.0 mM enhanced rather than inhibited the ATPase. Above 10^{-5}M Ca^{2+} cardiac myofibril ATPase activity was lowest in the 0.04 Mg^{2+} condition. In both the cardiac and skeletal myofibrils there was little difference in the effects of 5 and 10 mM Mg^{2+}. Normalization of ATPase data revealed a clear Mg^{2+}-induced rightward shift but also revealed that this effect was much more pronounced in cardiac ATPase between 1 and 5 or 10 mM Mg^{2+}. Reducing Mg^{2+} to 0.04 mM caused a leftward shift in the pCa -ATPase curve of similar magnitude in cardiac and skeletal samples (Solaro and Shiner, 1976).

The mechanism proposed to account for the effects of Mg^{2+} on Ca^{2+} activation involves interaction of Mg^{2+} with Ca^{2+}-binding sites on the myofibrils (Solaro and Shiner, 1976; Potter and Gergely, 1975). Divalent cation binding sites are located on most of the myofibril proteins. In particular the high affinity Ca^{2+}-Mg^{2+} and Ca^{2+}-specific sites on troponin (Potter and Gergely, 1975; Johnson et al., 1979) and the high affinity sites on myosin (Bagshaw, 1977) have been implicated in the apparent regulation by Mg^{2+}. The study of Solaro and Shiner (1976) examined myofibrillar-bound Ca^{2+} and its relationship to ATPase at different Mg^{2+} levels. At Ca^{2+} concentrations exceeding 10^{-6}M, Ca^{2+} binding increased to a significant degree in the lowest Mg^{2+} condition (0.04 mM) in both skeletal and cardiac groups. Dissimilar responses were observed where an increase from 1.0 to 10.0 mM Mg^{2+} slightly reduced skeletal myofibril Ca^{2+}-binding while it enhanced that of cardiac myofibrils, particularly at high Ca^{2+} (10^{-5}M). This enhanced myofibrillar Ca^{2+}-binding was attributed to enhanced binding by troponin since Ca^{2+}-binding to myosin was greatly depressed. Low Mg^{2+} resulted in a large increase of myosin-bound Ca^{2+}. It was suggested that the inhibition of cardiac ATPase activity at 0.04 mM Mg^{2+} could be attributed to the increase of Ca^{2+} on myosin (Solaro and Shiner, 1976). This observation corresponds to that of Bremel and Weber (1975) where micromolar Mg^{2+} depressed the actomyosin ATPase activity of a troponin-free system as Ca^{2+} increased from 10^{-7} to 10^{-5}M. The Ca^{2+} requirements for activation of cardiac myofibrils exhibit distinct differences at different Mg^{2+} levels. In addition while significant Ca^{2+}-binding was observed to occur at very low ATPase activity at 0.04 mM Mg^{2+}, at 1.0 and 10.0 mM Mg^{2+}, the relationship between ATPase and binding was very steep.

At Ca^{2+} concentrations below 2 x 10^{-6}M, the lack of an effect of high Mg^{2+} on Ca^{2+}-binding despite a depression in the ATPase activity led Solaro and Shiner (1976) to suggest a role for Mg^{2+}-specific sites shown to exist on troponin-C (Potter and Gergely, 1975). However, the results at higher Ca^{2+} become more difficult to interpret. The original suggestion that simple competition between Mg^{2+} and Ca^{2+} for binding at the troponin high-affinity sites has been dispelled by several studies. The non-uniform effects of low versus high Mg^{2+} on cardiac myofibril Ca^{2+}-binding were the first indication of a more complex mechanism (Solaro and Shiner, 1976). Potter et al. (1981) further demonstrated, using a fluorescent-label on the Ca^{2+}-specific site of TnC, that very high Mg^{2+} concentrations, deemed unphysiological, noncompetitively reduced the affinity of Ca^{2+}-specific sites for Ca^{2+}. However within a physiological range of 1-2 mM, Mg^{2+} did not have a significant effect on these sites. They suggested that this effect of high Mg^{2+} may explain the earlier observations of reduced Ca^{2+}-sensitivity of tension and ATPase. This does not explain the enhancement of Ca^{2+}-binding at 10 mM Mg^{2+} observed by Solaro and Shiner (1976) in cardiac myofibrils. With respect to the high-affinity Ca^{2+}-Mg^{2+} sites it was determined that the on and off rates of both cations was too slow to be of importance in the Ca^{2+}-

activated switching mechanism (Potter et al., 1981; Robertson et al., 1981; Johnson et al., 1981). However, high Mg^{2+} levels did depress the Ca^{2+} affinity of these sites to a large extent. With repeated Ca^{2+} transients Ca^{2+} saturation only rose to 35% (Potter et al., 1981). The significance of these sites in muscle contraction has not yet been defined and therefore an understanding of regulation at these sites remains out of grasp. Proposals include regulation of troponin conformation and thereby indirect regulation of Ca^{2+}-binding to Ca^{2+}-specific sites (Potter et al., 1981). Mg^{2+} induces similar conformational changes in troponin as Ca^{2+} upon binding to the Ca^{2+}-Mg^{2+} sites therefore regulation may involve a Ca^{2+}-Mg^{2+} exchange. Thus, the possibility that Ca^{2+} exchange rates at Ca^{2+}-specific sites may vary depending on the relative amounts of Ca^{2+} and Mg^{2+} bound to high-affinity sites is another proposed mechanism for Mg^{2+} influence (Robertson et al., 1981). Furthermore, Holroyde et al. (1980) observed that the positive cooperativity of Ca^{2+}-binding to the high-affinity sites was depressed by Mg^{2+} which does not exhibit this binding characteristic.

The influence of Mg^{2+} quite possibly extends to myosin as well since myosin contains two Ca^{2+}-Mg^{2+} sites on the P-light chain (Holroyde et al., 1979[b]). The existence of a Ca^{2+}-regulatory mechanism on myosin has been proposed (Lehman, 1978). In the presence of 0.30 mM Mg^{2+} the Ca^{2+} affinity of the two myosin sites was reduced by almost two orders of magnitude. Thus, based on the assumption of simple competition for binding, the similar affinities of Mg^{2+} and Ca^{2+} for myosin sites when present together reduces total bound Ca^{2+} (Holroyde et al., 1980). Since myosin cation exchange has also been shown to be very slow any effect of Mg^{2+} would not account for regulation of rapid Ca^{2+}-dependent activation (Robertson et al., 1981). However, a long-term modulation of mechano-chemical activity has been hypothesized (Rupp, 1980).

pH

Pathophysiological states such as ischemia, hypoxia and acidosis have been shown to elicit changes in the contractile properties of the myocardium as well as in skeletal muscle (Rupp, 1980). With regard to myocardial ischemia, intracellular acidosis has been shown to provoke a direct and pronounced negative inotropic effect on tension generation at the myofibril level and may be responsible for the early, rapid decline in force generation observed in ischemic conditions (Donaldson and Hermansen, 1978; Fabiato and Fabiato, 1978; Rupp, 1980). Evidence that excess H^+ acts primarily intracellularly was obtained by Poole-Wilson and Langer (1975) where respiratory acidosis (increased PCO_2) and metabolic acidosis (decreased HCO_3^-) were induced in a skinned fiber preparation. A greater and more rapid decline in force production was obtained in the elevated PCO_2 condition since gases equilibrate across membranes more rapidly than do large anions such as HCO_3^-; moreover, the greater gas effect also caused a transient negative inotropic effect even when extracellular pH in the bathing solution was maintained at normal by increasing HCO_3^- concentration (Poole-Wilson and Langer, 1975).

Normal intracellular pH in the myocardium has been measured by the chemical shift of Pi in ^{31}P NMR spectroscopy and was shown to be between 7.22 ± 0.02 (Jacobus et al., 1982) and 7.0 (Salhany et al., 1979). In vivo, the cytoplasmic pH is the sum of a number of processes involving consumption and production of protons such as glycolysis, oxidative phosphorylation and related reactions, CO_2 production, creatine phosphate consumption, glycogen metabolism, lipid metabolism and cation and anion exchanges across the cell membrane (Gevers, 1977). It has also been suggested that contrary to popular thought, lactate production may not be the primary determinant of cell pH (Salhany et al., 1979); rather, in the heart, ATP hydrolysis may be the major source of direct cytoplasmic proton production (Gevers, 1977). Therefore, processes intimately involved in and dependent upon ATP hydrolysis such as E-C coupling and myofibril contraction, may be directly influenced by excess H^+. Despite uncertainty as to the sources of H^+ in intracellular acidosis, the effects on myofibril tension generation, Ca^{2+} homeostasis, myofibril ATPase activity and Ca^{2+}-sensitivity are now well-documented.

The effect of intracellular acidosis on tension generation has been examined in chemically and mechanically-skinned cardiac and left ventricular fibers as well as in skinned skeletal muscle preparations from rabbit, rat and frog models. Maximal and submaximal levels of isometric force production with varying free Ca^{2+} concentrations over a pH range of 6.2 to 7.4 were employed (Donaldson and Hermansen, 1978; Fabiato and Fabiato, 1978; Donaldson et al., 1981). An acidotic depression in maximal force was demonstrated for both cardiac and skeletal muscle where a decrease to pH 6.5 resulted in a ratio of maximal tension at pH 6.5 to that at 7.0 of 0.82 ± 0.004 in cardiac fibers (Donaldson and Hermansen, 1978). A pH change from 7.4 to 6.2 similarly reduced maximal tension in cardiac and skeletal fibers at high free Ca^{2+} concentrations (Fabiato and Fabiato, 1978). The most dramatic responses to elevated H^+ are seen in the Ca^{2+}-sensitivity or pCa-tension curves especially of cardiac muscle where pCa-tension curves become displaced to the right with lowered pH (pCa = the negative log of Ca^{2+} concentration) (Donaldson et al., 1981; Fabiato and Fabiato, 1978). Submaximal tension appeared to be more susceptible to pH alterations in cardiac versus skeletal muscle where the free Ca^{2+} concentrations required for 50% activation increased by a factor of 5.5 versus 3.47 respectively with a pH decrease to 6.20 (Fabiato and Fabiato, 1978). The effects of pH were reversible but could not be overcome by simply increasing Ca^{2+} concentration which suggests an indirect interaction between H^+ and Ca^{2+} ions at myofibril sites (Fabiato and Fabiato, 1978). Absolute force generation of cardiac and adductor fibers, but not of soleus fibers, was significantly depressed over the full range of Ca^{2+} (pCa 7.75 to 4.0) regardless of their respective levels of activating Ca^{2+} (Donaldson and Hermansen, 1978). However, the cooperativity or general steepness of the pCa-tension relationship, as expressed by the Hill n value, does not seem to be influenced by pH (Rupp, 1980; Donaldson et al., 1981). In addition to skinned fiber or disrupted bundle models, work with normal isovolumic perfused rabbit heart has demonstrated that a 0.22 pH unit acidification correlated with a 50% depression in left ventricular developed pressure (LVDP). In the ischemic model, 50% depression in LVDP correlated only with a 0.09 unit reduction in pH which led the authors to suggest that other factors influenced contractility in ischemia since the pH change was only 40 to 50% of that in "normal" acidosis (Jacobus et al., 1982).

Studies on the pH-sensitivity of myofibril ATPases of cardiac and skeletal muscle have further demonstrated the negative inotropic effect of intracelluar acidosis on muscle force-generating capabilities (Okabe and Hess, 1981; Kentish and Nayler, 1979; Portzehl et al., 1969). Basal myofibril ATPase activity (pCa < 7.5) has shown variable responses to acidosis. Rabbit basal cardiac ATPase was unaffected over a pH range of 6.4 to 7.2 (Kentish and Nayler, 1979) but was later shown to be significantly depressed at pH 6.4 but not between 6.8 and 7.2 (Okabe and Hess, 1981). In fast skeletal muscle the basal ATPase activity is mildly affected by pH where slightly higher activity was recorded under mild alkylotic conditions (pH 7.2) (Kentish and Nayler, 1979). Maximally-activated (pCa 4.5) cardiac ATPase activity decreased by 20% when pH was reduced from 7.0 to 6.5 while no alteration was observed in skeletal ATPase (Kentish and Nayler, 1979). As in the skinned fiber preparations, cardiac and skeletal pCa-ATPase relationships were markedly shifted to the right with intracellular acidosis. A decrease in pH of 0.2 units lowered the pCa for half-maximal activation by about 0.2 pCa units in both muscle types, while the pCa values for threshold and maximal activation were altered to a similar degree. Thus, a reduction in pH from 7.20 to 6.40 produced a 4-fold increase in the Ca^{2+} concentration required for half-maximal activation in skeletal ATPase and a 5-fold increase for cardiac ATPase (Kentish and Nayler, 1979). This agrees with the quantitative relationships for tension generation shown by Fabiato and Fabiato (1978) and is about double those found by Donaldson and Hermansen (1978). In accord with this research, Okabe and Hess (1981) showed an increasing sensitivity to pH with increasing Ca^{2+} concentrations in cardiac muscle. At a pCa of 7.0 a pH change from 7.2 to 6.8 did not affect the ATPase while at a pCa of 6.65 the ATPase became depressed at pH 6.8, at pH 6.6 the pCa required for 50% activation changed from 6.6 to 6.3 (higher Ca^{2+}) and at pH 6.4, the basal 50%-activated and V_{max} ATPases were all depressed. The similarity in the responses of tension generation in fibers and myofibril ATPase activities to intracellular acidosis strongly support an interaction of H^+ with the contractile proteins and regulation by Ca^{2+}.

The influence of excess H^+ in combination with other regulatory elements in the myofibrils, Mg^{2+} and $MgATP^{2-}$, has also been investigated.

With regard to Mg^{2+}, at 1 mM Mg^{2+} (physiological) the typical acidotic effect on tension generation was observed while at 10 mM Mg^{2+}, Ca^{2+}-sensitivity of tension was unaffected at pH 6.5. Thus, the differential depressant effect of H^+ on Ca^{2+}-sensitivity appears to be Mg^{2+}-dependent (Donaldson and Hermansen, 1978). Depletion of $MgATP^{2-}$ has also been considered a possible factor in the reduced contractility seen with ischemia and/or acidosis. Reduction of $MgATP^{2-}$ to 0.1 mM in cardiac skinned fibers did not further influence the acidotic reduction in force generation and Ca^{2+}-sensitivity (Donaldson et al., 1981). The enhnaced responses at normal pH to extremely low $MgATP^{2-}$ (0.03 mM) (Orentlicher et al., 1977) however were abolished at pH 6.5 which led to the implication of an H^+-$MgATP^{2-}$ interaction in acidotic negative inotropy (Donaldson et al., 1981).

While it is evident that H^+ directly influence the contractile proteins and their regulation, the mechanisms and sites of this influence remain obscure. One mechanism frequently implicated in the acidotic effect is the direct competition between H^+ and Ca^{2+} for binding to troponin C (TnC) (Rupp, 1980). However, this mechanism is highly controversial in terms of the type of interaction between H^+ and Ca^{2+} at the TnC sites (competitive versus noncompetitive; direct versus indirect), the particular sites of the interaction (low versus high-affinity) and the appropriateness of the site itself. Fuchs (1979) demonstrated that variation of pH over the range of 6.2 to 7.4 had no effect on Ca^{2+}-binding to glycerinated rabbit psoas muscle fibers in the presence of 1 mM Mg^{2+}. However, the possibility that H^+-Ca^{2+} competition may only occur at the low-affinity, Ca^{2+}-specific sites on TnC was addressed by Robertson et al., (1978[a] and [b]). These authors showed through fluorescent enhancement of labelled TnC from rabbit skeletal and bovine cardiac muscle that the Ca^{2+}-specific, but not the Ca^{2+}-Mg^{2+}, sites were pH sensitive. Other work utilizing Ca^{+} papillary muscle demonstrated reduced developed tension and rate of rise of tension with decreased pH (7.6 to 7.0) but discounted direct H^{+}-Ca^{2+} ion competition at TnC suggesting instead a noncompetitive interaction between the two cations (Mattiazzi et al., 1979). Thus the question of H^{+} action on TnC remains unsettled. Reduced pH probably results in the protonation of a large number of functional groups on the contractile proteins and therefore other sites of H^{+} influence may include tropomyosin conformation and troponin subunit interaction (Fabiato and Fabiato, 1978). In addition, recent work has shown that the S-2 region on myosin is readily affected by small alterations in charge balance where conformation changes in this region would lead to a change in the disposition of the myosin heads and hence to altered force production (Reisler and Liu, 1982).

Interruption in the E-C coupling process, specifically Ca^{2+} transport by the SR, has also been implicated in the acidotic depression of contractility (Fabiato and Fabiato, 1978; Okabe and Hess, 1981). The amplitude of caffeine-induced contractures was used to study Ca^{2+}-loading in the SR of skeletal and cardiac skinned fibers (Fabiato and Fabiato, 1978). In skeletal fibers, the pH optimum for Ca^{2+}-loading depended on the free Ca^{2+} concentration where loading at lower free Ca^{2+} levels (pCa 7.75) had higher pH optimums (pH 7.0 to 7.4). Therefore, mild acidosis increased loading at higher free Ca^{2+} levels (pH 7.0 to 6.6 at pCa 7.00 and pH 6.6 to 6.2 at pCa 6.00). Contraction amplitudes were significantly depressed in skeletal fibers when the pH of the loading solution was either reduced to 6.2 or raised to 7.4, with the greater effect at pH 6.2. In the cardiac preparation similar contraction amplitudes and pH optima were observed at a pCa of 7.75. However, at lower pCa's cardiac and skeletal responses were radically different. A change in pCa from 7.75 to 7.0 in the loading solution led to a decrease or no change in the caffeine-induced contractions of cardiac fibers. Those results were attributed to a Ca^{2+}-induced Ca^{2+} release from the SR (Fabiato and Fabiato, 1978). An increase in the free Ca^{2+} concentration to a pCa of 6.5 was required in the loading solution at pH 6.20 or 6.60 in order to achieve a similar level of Ca^{2+}-induced Ca^{2+} release which supports the theory of a graded response of Ca^{2+} release from cardiac SR (Fabiato and Fabiato, 1978 and 1979). Thus, in cardiac SR the pH optimum for Ca^{2+} loading is in the alkaline range and acidosis appears to reduce the loading as well as require a greater Ca^{2+} stimulus for Ca^{2+} release (Fabiato and Fabiato, 1978). A significant decline in the amount and velocity of Ca^{2+}-uptake in cardiac SR over a pH range of 7.0 to 6.4 concurs with the above data where also the efficiency of Ca^{2+} transport (ratio of μ moles Ca^{2+} transported to μ moles ATP hydrolyzed) declined from 0.87 at pH 7.0 to 0.637 at pH 6.6 and to 0.507 at pH 6.4 (Okabe and Hess, 1981). In summary then the consequences of decreased pH in cardiac SR were depressed Ca^{2+} loading, depressed rate of Ca^{2+} accumulation and also depressed Ca^{2+}-induced Ca^{2+} release.

The depression in SR function by acidosis is probably part of the mechanism for the decrease in both contraction amplitude and relaxation rate observed in ischemic, acidotic cardiac tissue (Mattiazzi et al., 1979); this impaired function therefore accentuates the depressive effects of acidosis on the myofilaments. A significant difference between the cardiac and skeletal muscles is that the acidotic effects on skeletal SR may tend to compensate for the depressive effects on the myofilaments (Fabiato and Fabiato, 1978; Okabe and Hess, 1981). Thus acidosis in cardiac muscle profoundly influences its function and is likely a primary determinant of contractility (Donaldson et al., 1981).

Inorganic Phosphate

Inorganic phosphate is a proton-donating anion and has recently been shown to participate in the regulation of ionic exchange, the cellular energetic state and in contractile function (Ponce-Hornos and Langer, 1983). The ionization of inorganic phosphate (Pi) is pH-dependent and proceeds as $H_3PO_4 \longleftrightarrow H_2PO_4^- + H^+ \longleftrightarrow HPO_4^{2-} + 2H^+ \longleftrightarrow HPO_4^{3-} + 3^+$ (Ponce-Hornos et al., 1982). The oxidation state of Pi may determine its effect on cellular function.

Recently, Pi was shown to influence Ca^{2+} compartmentalization in cultured heart cells (Ponce-Hornos et al., 1982). The presence of 10 mM Pi stimulated mitochondrial Ca^{2+}-uptake particularly below pH$\sim$7.2. Above pH 7.2 the increase of PO_4^{3-} concentration activated another mechanism which augmented sarcolemmal Ca^{2+} uptake via a Pi-dependent inhibition of the Na^+-K^+ pump and a secondary activation of Na^+-Ca^{2+} exchange. Thus, a major effect of $pH > 7.2$ in the presence of 10 mM Pi was to activate a non-mitochondrial PO_4^{3-}-concentration-dependent sarcolemmal mechanism for Ca^{2+} uptake. This augmentation of the inward movement of Ca^{2+} was suggested to bear some significance with regard to the role of extracellular Ca^{2+} in activating the myofibrils and also as a trigger of Ca^{2+} release from the SR (Ponce-Hornos et al., 1982).

Inorganic phosphate has also been implicated in regulation at the level of the SR and myofibrils as well. Increasing Pi concentration from 0 to 20 mM in a bath containing cardiac fiber bundles resulted in a 50% decrease in isometric force and immediate stiffness in quick release experiments (Herzig and Ruegg, 1977). Similar results were obtained with 10 mM Pi while the V_{max} was also observed to decrease (Herzig and Ruegg, 1980). Additional studies have also employed vanadate, a transition state analog of phosphate which is considered to be a useful probe of the myosin ATPase mechanism (Goodno, 1970; Solaro et al., 1980[a]). Micromolar effects of vanadate (Vi) are similar to those of millimolar Pi (Solaro et al., 1980[a]). Herzig et al. (1981) demonstrated that 20 μM Vi and 10 mM Pi both elicited a decrease in isometric tension and immediate stiffness of isolated cardiac muscle. In addition, Pi and Vi activated ATPase activity of the fiber bundles at any particular level of tension development above basal. The increased ATPase activity and increased rate of delayed tension development were interpreted as an effect of Pi and Vi on the kinetics of cross-bridge cycling where rates become enhanced. Thus the cross-bridges spend less time attached to actin and therefore in active force generation. The effect of these ions would then involve a greater ATP cost of tension maintenance (Herzig et al., 1981). Vanadate inhibited isometric tension and stiffness of chemically-skinned heart muscle fibers with a Ki of 50 μM but did not influence Ca^{2+}-binding or Ca^{2+}-dependence (Solaro et al., 1980[a]). The effects of Pi have also been shown to be Ca^{2+}-independent (Herzig and Ruegg, 1980). Micromolar Vi and millimolar Pi both elicited depressions in myofibril ATPase activities over a range of activating Ca^{2+} (Solaro et al., 1980[a, b]). Furthermore, Vi inhibited the SR ATPase, the rate of oxalate-supported Ca^{2+} uptake by SR and the myofibril ATPase activity with Ki of 50 to 70 μM (Solaro et al., 1980[b]). Interestingly, Vi was shown to exert a positive inotropic effect on the left ventricular pressure and force development of whole isolated perfused hearts with no effect on relaxation parameters (Solaro et al., 1980[b]). Vi also decreased the extent of TnI phosphorylation which would also contribute to the positive inotropic response. The positive and negative inotropic effects of Vi were suggested to involve multiple sites in the myocardial cell which could depend on its state of oxidation (Solaro et al., 1980[b]).

Contribution of Pi to regulation of contractile activity of the heart may become more important under certain conditions. Pi accumulation has been observed in heart muscle during ischemia and in skeletal muscle during fatigue (Furchgott and Lee, 1961; Jacobus et al., 1977). In addition Pi may accumulate intracellularly when the ratio of CP to C decreased during inotropic stimulation (Herzig and Ruegg, 1977).

IV. CARDIOVASCULAR AND MYOCARDIAL EFFECTS OF ENDURANCE EXERCISE

Endurance exercise has been shown to enhance cardiac performance in both humans and experimental animals (Scheuer and Tipton, 1977). In humans the major cardiovascular adaptations to chronic endurance exercise include resting bradycardia, submaximal exercise bradycardia, increased stroke volume (SV) at any level of exercise, increased cardiac output (CO), increased maximal oxygen consumption (max $\dot{V}O_2$) and an increased arterio-venous O_2 difference (a-v O_2 difference) (Holloszy, 1976; Scheuer and Tipton, 1977). Peripheral vascular and subcellular adaptations in skeletal muscle have also been widely documented and contribute to overall endurance performance. Although skeletal muscle adaptations are exercise- and fiber-type-specific in general, chronic endurance exercise has been shown to elicit increased mitochondrial size, number and density, increased quantity and activity of TCA cycle enzymes, respiratory chain enzymes involved in NADH oxidation and ATP synthesis and enzymes responsible for activation, transport and catabolism of fatty acids, an increased capacity to utilize substrate parallelled by an increased capacity to generate ATP oxidatively, an increased myoglobin content and O_2 delivery capability and increased blood flow to working muscles. No major changes are reported within the glycolytic pathway (Holloszy, 1976). Cardiovascular and myocardial adaptations to endurance exercise will occupy the focus of this section with occasional contrasts to accompanying skeletal muscle adaptations.

THE EXERCISE STIMULUS

Both swimming and running have been employed as chronic endurance exercise stimuli to investigate cardiac adaptations as well as those occurring in skeletal muscle. Evidence suggests that the nature of the stimulus may differ between modes of exercise in both acute and chronic situations since the types and extent of cardiovascular and subcellular adaptations reported in the literature are not congruent (Flaim et al., 1979; Schaible and Scheuer, 1979; Baldwin et al., 1977). Common criteria for the evaluation of the magnitude of the exercise response are heart rate (Baldwin et al., 1977) and oxygen consumption (Baker and Horvath, 1964; McArdle, 1967). Therefore, it is important to first examine these indices of metabolic stress in both swimming and running before proceeding to study other adaptive mechanisms. Factors which influence the training effects of both exercises are frequency, intensity, duration, age of onset, sex and strain of animal (Scheuer and Tipton, 1977; Dawson and Horvath, 1970).

The Albino rat is the most frequently used model in swimming studies. Quantitative assessment of energy expenditure is difficult in swimming and is subject to variation due to water temperature, weighted versus unweighted conditions, type of chamber, psychological stress, learning and number of animals per chamber (McArdle, 1967; Dawson and Horvath, 1970; Scheuer and Tipton, 1977).

Maximum swim times are obtained at water temperatures at or slightly below the animal's core temperature (about 37°C) while temperatures above or below this narrow range reduce performance (Dawson and Horvath, 1970). Naive rats swum at 37°C with rectal probes were able to continue for 45 minutes while those swum at 20° or 42°C could not complete the duration. Performance duration was related to deviation of core temperature from normal (Baker and Horvath, 1964). At 37°C, these rats achieved $\dot{V}O_2$'s of 30 to 37 $ml \cdot kg^{-1} \cdot min^{-1}$ (Baker and Horvath, 1964).

OXYGEN CONSUMPTION ($\dot{V}O_2$)

The metabolic requirements of unweighted swimming in rats are relatively mild due to the animal's natural buoyancy accruing from a high relative body fat and the dead-air space in fur (Dawson and Horvath, 1970). Resting $\dot{V}O_2$ in rats has been reported in the range of 14 to 24 $ml \cdot kg^{-1} \cdot min^{-1}$ (Baker and Horvath, 1964; McArdle, 1967; Shepherd and Gollnick, 1976). In order to increase exercise intensity uniformly in animals of varying size, a percentage of body weight is often attached to the tail. However, this method may still favour lighter weight animals due to the lesser absolute load (Dawson and Horvath, 1970). McArdle and Montoye (1966) found that swim times were significantly decreased in heavier rats despite equal relative tail weights. In an acute situation swimming rats with rectal probes corresponding to approximately 3% of body weight attained $\dot{V}O_2$'s of about 3 times basal values or 45 $ml \cdot kg^{-1} \cdot min^{-1}$ (Baker and Horvath, 1964). The effects of a mild 4 week training program, where rats swam unweighted 5 days per week for up to 30 minutes per day until addition of 4% body weight in week 4, were evaluated during a ten minute swim and 25 minute recovery. Animals with 0 load increased their $\dot{V}O_2$ 2.7 times above rest (to 62.6 $ml \cdot kg^{-1} \cdot min^{-1}$) while incremental increases in $\dot{V}O_2$ were observed with 0.5, 1.0 and 1.5% body weight finally reaching 80.86 $ml \cdot kg^{-1} \cdot min^{-1}$ with 2.0% body weight. At the heaviest load, recovery required 25% more time than unweighted animals (McArdle, 1967). While this study did not distinguish between the acute effects of increased intensity and a chronic adaptation of $\dot{V}O_2$ to the 4 week program, it did provide some insight into possible differences in $\dot{V}O_2$ and performance depending on the ability to remain at the surface. Animals that experienced difficulty in swimming at the surface as tail weights were added were unable to reach levels of aerobic metabolism attained by the others. In addition, the $\dot{V}O_2$ of these animals, while not reaching as high levels, remained elevated longer during recovery (McArdle, 1967). Thus, it has been suggested that weighted swimming where submersion occurs more frequently may stimulate anaerobic metabolism in some animals at least acutely (McArdle, 1967; Dawson and Horvath, 1970). Blood lactate data to support this theory is diffuclt to interpret due to lack of uniform conditions across studies (Dawson and Horvath, 1970).

Treadmill running studies have used both rodents and dogs. Unlike aquatic exercise, the rate and magnitude of the workload are precisely controlled by varying the speed and grade of the treadmill and total distance and work can be quantified (Flaim et al., 1979; Scheuer and Tipton, 1977). The major physical variables influencing performance, at least acutely, are age and body weight (Flaim et al., 1979).

In examining the acute $\dot{V}O_2$ response to treadmill running at various temperatures in animals acclimatized to different temperatures Pasqui et al. (1970) found that rats acclimatized to and running in 30°C at a speed of 77 $m \cdot min^{-1}$ with a 19% incline achieved a $\dot{V}O_2$ max of 76.5 $ml \cdot kg^{-1} \cdot min^{-1}$. Animals acclimatized to lower temperatures but running at 30°C demonstrated greater $\dot{V}O_2$ max. Male Sprague-Dawley rats, trained for 6 weeks on a program of running at 28 $m \cdot min^{-1}$ for 60 minutes, 5 days per week in a work wheel, demonstrated increases in $\dot{V}O_2$ as a linear function of work intensity until max. Between 49 and 67 $m \cdot min^{-1}$ the animals obtained $\dot{V}O_2$ max of 93 to 95 $ml \cdot kg^{-1} \cdot min^{-1}$ (Shepherd and Gollnick, 1978). In addition, a pattern of adjustment to submaximal steady-state exercise similar to that of man was observed. A max $\dot{V}O_2$ test on the treadmill comparing trained and untrained female rats revealed that the trained group achieved significantly higher $\dot{V}O_2$ than untrained (81.5 ± 1.7 versus 71.6 ± 0.94 $ml \cdot kg^{-1} \cdot min^{-1}$) and also attained greater peak running speeds. In addition, at submaximal levels the trained runners performed at a lower percent $\dot{V}O_2$ max than did the untrained group (Patch and Brooks, 1980). Thus the adaptations of oxygen consumption in response to acute and chronic endurance exercise in rats are qualitatively similar to that observed in humans (Scheuer and Tipton, 1977).

HEART RATE

Acute heart rate responses to continuous exercise apparently differ between treadmill running and swimming. In naive rats subjected to a 15 minute bout of either running (22 $m \cdot min^{-1}$; 0° incline) or swimming (3% B.W.; 33°C) the heart rates of the running group increased significantly from rest (380 ± 10 bpm) to 480 ± 30 bpm, while those of the swimmers did not differ significantly from the resting value of 376 ± 15 bpm (Flaim et al., 1979). These results may not be comparable since of the intensities of the two exercises, the weighted swimming was probably more severe based on comparisons between $\dot{V}O_2$ data from other studies (McArdle, 1967; Shepherd and Gollnick, 1976; Dawson and Horvath, 1970). In addition, the rats used in this study were very mature (> 600 g body weight) and it has been suggested that older animals respond less favourably to the stress of exercise, this aging effect being amplified as exercise intensity increases (Scheuer and Tipton, 1977; McArdle, 1967). Another group of naive rats swum for 10 minutes with approximately 3% B.W. at 37°C obtained heart rates of 480 bpm which decreased in water temperature of 28°C and increased to 520 bpm in 42°C water (Baker and Horvath, 1964).

Endurance training by either running or swimming elicits resting bradycardia (Carey et al., 1979; Dowell et al., 1977; Schaible and Scheuer, 1979; Fuller et al., 1981). Swimmers were observed to have slightly lower resting heart rates versus runners (Schaible and Scheuer, 1979). Also comparable to the human adaptation is the lower submaximal heart rate in trained animals at any level of exercise (Dowell et al., 1977). After 8 weeks of endurance running (at 28 $m \cdot min^{-1}$; 8% incline) or swimming (unweighted; 180 $min \cdot day^{-1}$), Baldwin (1982) reported maximal heart rates of 570 bpm in the runners while those of the swimmers were observed to decrease by up to 100 bpm from resting levels. This response was attributed to possible diving bradycardia, a vagal reflex upon partial or complete submersion. However, another comparative study reported that trained runners achieved steady-state heart rates of 450 bpm after 20 minutes while swimmers ranged between 420 and 400 bpm, the heart rate declining gradually over this range during the exercise session (Schaible and Scheuer, 1979). The mechanism for exercise bradycardia with training is not well understood but appears to relate to adaptations in autonomic control where parasympathetic activity in the heart may be enhanced and that of the sympathetic system attenuated. Circulating catecholamines, elevated stroke volume and/or changes in integration patterns by the CNS may also contribute (Scheuer and Tipton, 1977).

ACUTE CARDIAC OUTPUT, DISTRIBUTION AND ARTERIAL PRESSURE RESPONSES

Naive rats in response to a 15 minute exercise bout demonstrated significantly elevated cardiac output (CO) with running and no change with weighted swimming. The heart rate component accounted for the increase in CO since no change in stroke volume (SV) was observed in either exercise (Flaim et al., 1979). However, in another swimming study CO increased from 300 to 460 $ml \cdot kg^{-1} \cdot min^{-1}$ and stabilized for 30 minutes of exercise at 37°C. Only a slight increase in SV was observed in the untrained animals (Dawson and Horvath, 1970). A marked difference in cardiac output distribution between acute swimming and running exposures, resulted in significantly elevated skeletal muscle and coronary artery blood flows in running while a lesser increase in skeletal muscle flow and no significant change in coronary blood flow occurred in swimming (Flaim et al., 1979). Neither exercise elicited a significant change in arterial pressure in the study of Flaim et al. (1979) while Dawson and Horvath (1970) have reported increases in pressure with swimming at 37°C.

ENDURANCE TRAINING

1. MORPHOLOGICAL CHANGES

Chronic endurance exercise provokes changes in body weight and/or heart weight, the extent of which appear to be dependent on sex and mode of training. Females demonstrate less or no weight loss with endurance training and body weights tend to follow the normal growth curve (Scheuer and Tipton, 1977; Schaible et al., 1981). Significant reductions in body weights of males are commonly reported with both swimming and running protocols (Penparkgul et al., 1978; Schaible and Scheuer, 1979; Tibbits et al., 1978; Nutter et al., 1981; Giusti et al., 1978; Malhotra et al., 1976). However, in some programs not apparently different from those above, male rats retained a normal rate of weight gain (Baldwin et al., 1977; Hepp et al., 1974; Wilkerson and Evonuk, 1971). Studies employing dogs of both sexes and treadmill running have reported little change in body weight relative to sedentary animals (Barnard et al., 1980; Carey et al., 1979; Dowell et al., 1977).

With regard to heart weight, several studies have documented no significant alterations with endurance running in male rats (Tibbits et al., 1978; Schaible et al., 1981; Nutter et al., 1981; Resink et al., 1981; Penparkgul et al., 1980). Female rats undergoing a high-intensity, intermittent running program demonstrated significant increases in heart weight (Baldwin et al., 1977) whereas those on less intense, continuous protocols showed slight but non-significant elevated heart weights (Baldwin et al., 1977) or no change at all relative to sedentary controls (Schaible et al., 1981). Dogs of both sexes subjected to endurance running have demonstrated significant (Barnard et al., 1980) or slightly elevated (Dowell et al., 1977) heart weights. With endurance swimming male rats have exhibited varied alterations in heart weight, some studies reporting significant elevations (Hepp et al., 1974; Bhan et al., 1972; Schaible and Scheuer, 1979; Scheuer and Stetzoski, 1972) while others detected no marked changes (Penparkgul et al., 1978; Wilkerson and Evonuk, 1971; Penparkgul et al., 1980; Guisti et al., 1978; Malhotra et al., 1976). Female rats are not employed as often in swimming studies but studies doing so have shown significant increases in cardiac mass (Crews and Adlinger, 1967; Penparkgul et al., 1979; Scheuer and Tipton, 1977; Molé, 1978).

The ratio of heart weight to body weight is frequently employed as an index of myocardial hypertrophy (Hepp et al., 1974). Generally, in endurance swimming and running studies which have shown an increased ratio, this change has been attributed to decreased body weight rather than increased heart weight and therefore absolute cardiac hypertrophy was not observed (Penparkgul et al., 1978; Tibbits et al., 1978; Schaible et al., 1981; Penparkgul et al., 1977; Giusti et al., 1978; Malhotra et al., 1976). Studies able to demonstrate significant cardiac hypertrophy may have employed more intense training programs (Baldwin et al., 1977; Bhan and Scheuer, 1972; Hepp et al., 1974; Barnard et al., 1980; Schaible and Scheuer, 1979; Scheuer and Stetzoski, 1972) or used female rats (Penparkgul et al., 1979; Crews and Adlinger, 1967; Scheuer and Tipton, 1977; Molé, 1978).

2. CARDIAC PERFORMANCE AND HEMODYNAMICS

Enhanced cardiac performance in response to endurance training has been reported in the literature on several occasions (Schaible and Scheuer, 1979; Bersohn and Scheuer, 1977). However, the results of such studies are by no means conclusive since divergent results from studies employing different animal models, preparations and training protocols have emerged (Nutter et al., 1981) as well as differences reported between studies using a similar model, preparation and exercise (Fuller and Nutter, 1981; Schaible and Scheuer, 1979). The rat and dog models are most frequently employed in cardiac performance studies where performance has been monitored under chronically-instrumented, in vivo or open chest, in situ conditions as well as in the isolated, perfused working heart preparation. The two former techniques offer the advantage of a more authentic physiological response to the exercise stress while the latter model allows the researcher to observe intrinsic cardiac function free of any neurohumoral and peripheral vascular compensatory mechanisms. Also preload, afterload and heart rate can be controlled while different types of physiological stress can be imposed on the heart by manipulating these variables or by introducing hypoxic or ischemic conditions.

Both running and swimming regimens have elicited increases in some flow-related variables. Chronically-instrumented, endurance-run dogs showed elevated cardiac output (CO) (9.1 ± 0.7 versus 7.1 ± 0.5 $\mathrm{l \cdot min^{-1}}$) and stroke volume (SV) (32 ± 2.0 versus 25.9 ± 2.0 $\mathrm{ml \cdot beat^{-1}}$) relative to sedentary animals in response to a maximal treadmill test (Barnard et al., 1980) while a similar study concurred (Dowell et al., 1977). Endurance-run, isolated male rat hearts demonstrated significant elevations in CO and SV particularly in response to increased preload where atrial filling pressures were 10, 15 or 20 cm H_2O (Schaible and Scheuer, 1979; Schaible et al., 1981) while another study failed to reproduce these results with the same strain of rat and similar training program (Fuller and Nutter, 1981). Ejection fraction, an indirect measure of SV, was also increased at all filling pressures (Schaible and Scheuer, 1979). Endurance swimming has similarly produced elevated CO and SV, the differences above sedentary values increasing with increasing preload (i.e., at 20 cm H_2O: 540 versus 450 $\mathrm{ml \cdot min^{-1} \cdot g^{-1}}$; Schaible and Scheuer, 1979) (Penparkgul and Scheuer, 1970; Scheuer and Stetzoski, 1972; Bersohn and Seheuer, 1977; Giusti et al., 1978; Schaible and Scheuer, 1979). Enhanced left ventricular systolic pressure (LVSP) development and/or peak LVSP have been documented in some running and swimming studies where differences were more pronounced with higher preloads and basal levels often did not exceed those of control animals to a significant extent (Penparkgul and Scheuer, 1979; Bersohn and Scheuer, 1977; Schaible and Scheuer, 1979; Hepp et al., 1974). These adaptations have not been consistently demonstrated though as other swimming studies (Stetzoski and Scheuer, 1972; Giusti et al., 1978) and running protocols with rats or dogs (Fuller and Nutter, 1981; Schaible et al., 1981;

Barnard et al., 1980; Carey et al., 1976) did not reveal such alterations. An elevated maximal rate of pressure development (max + dP/dt) appears to be another accompaniment to endurance swimming (Penparkgul and Scheure, 1970; Hepp et al., 1974; Bersohn and Scheuer, 1977; Schaible and Scheuer, 1979) and has also been reported with endurance running (Dowell et al., 1977; Barnard et al., 1980). Again, as the workload on the heart increased, the max + dP/dt difference between trained and untrained animals enlarged. Studies which failed to show these differences included both types of exercise and some used similar ranges of preloads (Fuller and Nutter, 1981; Carey et al., 1976; Scheuer and Stetzoski et al., 1972; Giusti et al., 1978). Most of the cited research has also demonstrated significant increases in stroke work and/or maximum power of trained hearts with increasing preloads. It has been suggested that these latter variables, calculated from the product of pressure and flow, are more sensitive indicators of improved pumping capacity of the heart (Bersohn and Scheuer, 1977). However, one running study with rats did not demonstrate any change in stroke work (Fuller and Nutter, 1981). Schaible and Scheuer (1979) showed that increased stroke work with swimming was due equally to increased LVSP and to increased SV while in running it was 70% due to SV with less contribution from LVSP.

In order to determine whether the apparent improved pumping capacity is due to increased contractility as manifest by increased rate and extent of fiber shortening or rather to a Starling effect in response to increased end-diastolic volume (EDV) and increased ventricular compliance the EDV should be evaluated (Bersohn and Scheuer, 1977). The end-diastolic pressure (EDP) and EDP-EDV relationship provide further indication of the efficiency or energy cost of pump performance. In the absence of cardiac hypertrophy, EDV and EDP did not change in response to the swimming protocol of Bersohn and Scheuer (1977) and Schaible and Scheuer (1979) nor to the running programs of Schaible et al (1981). Another swimming study utilizing a slightly different protocol and different strain of rat (Sprague-Dawley versus Wistar) reported a 17% increase in cardiac mass which was accompanied by a shift to the right of the EDP-EDV curve. In this case EDV was increased for any given EDP while EDP was lower for any given EDV and was interpreted as an increase in cardiac efficiency (Hepp et al., 1974). Diastolic wall stress was only slightly increased for a given EDP in spite of the enlarged ventricular radius, while wall stress was reduced in the trained hearts at a given EDV. In addition the elastic stiffness at the midwall, a measure of the passive mechanical wall properties, was only slightly increased with training rather than decreased as would occur with ventricular dilation and pathological hypertrophy. Thus in this study the diastolic load on the LV myocardium was not increased; rather, an enhanced contractile capability appeared to occur (Hepp et al., 1974).

In the absence of cardiac hypertrophy and little change in EDV, the reported increases in circumferential fiber shortening, contractile element shortening and maximum rate of shortening supply further evidence for an adaptation of myocardial contractility to endurance exercise (Schaible and Scheuer, 1979; Barnard et al., 1980; Schaible et al., 1981).

An important adaptation where the rate of LV relaxation (max negative dP/dt) was enhanced has been widely reported in the endurance swimming studies (Bersohn and Scheuer, 1977; Giusti et al., 1978; Schaible and Scheuer, 1979) while the comparative study of Schaible and Scheuer (1979) and that of Barnard et al. (1980) illustrated that increased relaxation rates may not accompany running. Increased rates of myocardial relaxation with little reduction in the diastolic filling time would permit greater ventricular filling and hence contribute to the systolic performance.

Coronary flow is another variable related to myocardial performance and shown to be enhanced with training (swimming: Giusti et al., 1978; Penparkgul and Scheuer, 1970; Bersohn and Scheuer, 1977; Schaible and Scheuer, 1979). With running coronary flow was lower than that of swim-trained rats but still exceeded that of sedentary hearts (Schaible and Scheuer, 1979) or was shown not to differ from sedentary hearts (Fuller and Nutter, 1981). In chronically-instrumented dogs coronary flow tended to be lower during submaximal exercise in endurance-trained animals but was not different under maximal stress (Barnard et al., 1980). Myocardial oxygen consumption (m $\dot{V}O_2$) normally has a large reserve capacity but trends toward a greater capacity were reported in both running and swimming rats at higher preloads (Schaible and Scheuer, 1979; Giusti et al., 1978; Penparkgul and Scheuer, 1970; Bersohn and Scheuer, 1977).

The question of differences in adaptive mechanisms between male and female rats was addressed by Schaible et al. (1980). Endurance-run male rats demonstrated significant elevations in most of the aforementioned performance variables relative to their free-eating, sedentary counterparts and relative to the female runners. No significant differences were observed between female runners and controls. In order to determine whether the differential response was partly a result of smaller heart size in the females, they were compared to sedentary food-restricted males with similar heart size. However, it was found that these males demonstrated significantly greater values for stroke volume and stroke work. Thus, the lack of improved intrinsic performance of female hearts with endurance exercise, despite comparable increases in skeletal muscle oxidative capacity, suggest that sex hormones may mediate adaptive responses to exercise. In addition the authors acknowledged the possibility that the running regimen was of a lesser cardiovascular stress in the females and pointed to a lack of $\dot{V}O_2$ data on female rats to substantiate this point (Schaible et al., 1981).

Under hypoxic conditions where the PO_2 of the isolated heart perfusate is lowered to a value of 50% or less, endurance-trained hearts have demonstrated greater pump capacity (Carey et al., 1976; Scheuer and Stetzoski, 1972). In response to hypoxia performance variables including CO, SC, coronary flow, LVSP and max + dP/dt declined below baseline levels in both trained and sedentary hearts (Fuller and Nutter, 1981; Carey et al., 1976; Scheuer and Stetzoski, 1972). However, in an endurance-swimming study, CO, mean LVSP, coronary flow and calculated heart work were maintained at relatively higher levels by the trained hearts. In addition, as the hypoxic period continued, max + dP/dt and peak LVSP were observed to be slightly higher than in sedentary hearts. A small decrease in EDP with slightly higher peak LVSP suggested an increased contractile state in conditioned hypoxic hearts (Stetzoski and Scheuer, 1972). In endurance-run rat hearts the time required for a 50% reduction in myocardial performance in response to hypoxia was significantly greater than in sedentary hearts. Trained hearts maintained higher HR, LVSP, and HR-LVSP product throughout the hypoxic period and demonstrated a more rapid recovery with reoxygenation (Carey et al., 1976). Dissimilar results were observed by Fuller and Nutter (1981) with endurance running where hypoxia induced similar extents of reduced LVSP (28%) in trained and sedentary hearts. During recovery LVSP and CO were restored at comparable rates in the two groups while coronary flow recovery appeared more rapid in the trained group. In the left ventricular function curve of the relationship between LVEDP and myocardial stroke work index, the trained hearts demonstrated slightly greater stroke work for a given EDP than sedentary hearts. The lack of significant findings in Fuller and Nutter's paper (1981) may in part be due to the relatively shorter hypoxic period utilized (5 minutes versus 10 or 20 minutes) as compared to some other research (Scheuer and Stetzoski, 1972; Carey et al., 1976). Cutilleta et al. (1979) concluded from an 8 week running study that trained hearts are better able to tolerate increases in afterload and hypoxia, primarily through maintenance of stroke volume.

3. MYOCARDIAL MECHANICS

The improved pumping capacity of endurance-trained hearts and particularly the increased stroke volume implies enhanced contractility. To detdrmine whether there is an altered contractility, rather than a hypertrophy or increased Starling response, investigators have examined the contractile behavior of isolated strips of left ventricular papillary muscle. Thin papillary strips, which present little resistance to O_2 and substrate diffusion in the bathing medium, demonstrated no alteration in the passive length-tension relationship of male rat-hearts adapted to treadmill running (Nutter et al., 1981; Tibbits et al., 1978) and of female rat-hearts adapted to endurance swimming (Molé, 1978).
Contrary to these results, Meerson et al. (1980) demonstrated increased compliance in the papillary muscle strips of female, endurance-swum rat hearts where at equal resting tensions the adapted muscles had greater

lengths. The authors proposed that this adaptation would permit augmented ventricular diastolic volume and thereby implied a greater contribution of the Frank-Starling mechanism to cardiac performance with endurance swimming. The distinction between this study and the other three is not readily apparent since both types of exercise and both sexes yielded similar results in the first three. Both studies with female swimming rats resulted in hypertrophy (15% in Molé, 1978; 7% in Meerson et al., 1980).

The active length-tension relationship may also show adaptations to endurance training although the particular altered parameters may differ across studies (Tibbits et al., 1978; Meerson et al., 1980). Peak isometric tension was significantly greater in trained male rat hearts when stimulated at a rate of 0.5 Hz; normalization for cross-sectional area did not alter the result (Tibbits et al., 1978). The maximum active tension at L_{max} (length at which maximal tension occurs) was also significantly greater in female endurance-swum hearts and this difference was observed over the entire range of lengths studied (Molé, 1978). However, peak developed tension was not different in trained versus control hearts in other studies (Meerson et al., 1980; Nutter et al., 1981). Time-to-peak isometric tension (TPT) and half relaxation time (½ RT) were not influenced by running (Tibbits et al., 1978; Nutter et al., 1981) or swimming protocols (Molé, 1978). However, the maximum rate of tension development (max + dT/dt) was reported to be increased by 22% in female swim-trained hearts (Molé, 1978).

Force-velocity relationships exhibit adaptive responses to endurance training. An upward shift in the force-velocity curve with a significant but moderate 27% increase in the estimated V_{max} (unloaded shortening velocity) was observed with swimming (Molé, 1978). Increased velocities of isotonic contraction at light loads (Meerson et al., 1980; Molé, 1978) as well as elevated contraction amplitudes (Meerson et al., 1980) also appear to accompany endurance-swimming. Papillary muscle from treadmill-trained cats did not exhibit differences in isotonic contractile mechanics or in isometric responses relative to controls (Williams and Potter, 1976). These differences in the intrinsic myocardial responses may pertain to the nature of the exercise stimulus and/or to the animal model.

Although some studies have reported little change in half time of relaxation with running or swimming (Tibbits et al., 1978; Molé, 1978) another study was able to demonstrate a significant 20% increase in relaxation velocity over a range of frequencies from 60 to 300 contractions per minute (Meerson et al., 1980). Increased relaxation rates have been shown in isolated whole-heart preparations as well and may allow greater time for diastolic filling (Meerson et al., 1980; Schaible and Scheuer, 1979).

The inotropic response of papillary muscle strips to isoproterenol included comparable decreases in TPT and ½RT for control and trained hearts and no change in maximal tension. The relatively greater max + dT/dt of trained muscles was enhanced to an even greater degree than that of control hearts (Molé, 1978). In endurance-run hearts, bath norepinephrine levels did not differentiate between control and trained papillary muscles (Nutter et al., 1981). The degree and rate of development and decrement of the positive iontropic response to increased stimulation frequencies has also been reported to be significantly higher in endurance-swum rat hearts (Meerson et al., 1980). Altered bath Ca^{2+} concentrations failed to elicit a differential response in the trained papillary muscles with treadmill-running (Nutter et al., 1981).

Tibbits et al. (1978) investigated one possible mechanism for the increased contractility, that of Ca^{2+} interaction with components of the E-C coupling system. Perfusion of the papillary muscle strips with dantrolene, a skeletal muscle relaxant which inhibits Ca^{2+} release from the SR, did not differentiate between control and trained preparations. This suggested that intracellular Ca^{2+} uptake by SR may not be influenced by running (Tibbits et al., 1978; Sordahl et al., 1977). Lanthium (La^{3+}) was used to probe extracellular Ca^{2+} actions since it binds to anionic sites and displaces Ca^{2+} from the basement membrane while not permeating the cell membrane. A marked prolongation in the trained group in the time for decline to ½ tension suggested that more Ca^{2+} may be available from extracellular sites for the contractile system and may thus account for at least part of the observed increased myocardial contractility with training (Tibbits et al., 1978).

4. CONTRACTILE COMPONENTS (Myofibrils and SR)

Several studies examining the contractility of whole hearts and papillary muscle strips have speculated that altered myofibril ATPase activity may represent a cellular mechanism for the observed adaptations to chronic endurance exercise. Table A-2 presents a summary of research on the ATPase response to endurance training. In general the literature concurs that swimming induces increased ATPase activities (Bhan and Scheuer, 1972 and 1975 a,b; Malhotra et al., 1976; Giusti et al., 1978; Penparkgul et al., 1979; Rupp, 1981) while a concensus cannot be reached in regard to endurance running. A transient increase in Mg^{2+}-stimulated ATPase was observed but disappeared by 18 weeks (Baldwin et al., 1977). A more intense running program of intervals elicited a 15% increase in both Mg^{2+}- and Ca^{2+}-stimulated actomyosin ATPase activities (Baldwin et al., 1977) while the intense, long-duration program of Resink et al. (1981) consistently shows elevated Ca^{2+}-myosin ATPase activities with no change in the Ca^{2+}-Mg^{2+} actomyosin ATPase activity. However, the majority of running studies have failed to show altered ATPase activities (Table 1). The apparent differential effect on ATPase activity with swimming and running protocols may be related to the difficulty in comparing exercise intensities, to the possible differences in the exercises themselves, or to the particular in vitro system utilized (i.e., myosin versus actomyosin versus myofibrils). In addition to demonstration of a training effect through endurance swimming, periods of subsequent deconditioning have demonstrated that after 2 weeks of inactivity following 8 training weeks, actomyosin ATPase activity declined to levels comparable to the sedentary group (Malhotra et al., 1976; Giusti et al., 1978). A reduction in daily training time to 25% resulted in a decline of ATPase activity to sedentary levels by 30 days (Malhotra et al., 1976). The training and detraining responses of the actomyosin ATPase were parallelled by qualitatively similar responses of whole isolated heart cardiac output, coronary flow, myocardial $\dot{V}O_2$ and negative dP/dt (Giusti et al., 1978).

Elevated ATPase activity could be attributed to an increase in myofibril protein content, however this line of rationale is disputed by the results of some studies. Significant increases in the Ca^{2+}- and Mg^{2+}-actomyosin ATPases activities of male rat hearts subjected to a moderate endurance swimming protocol were not associated with evidence of hypertrophy (Bhan and Scheuer, 1972). Examination of protein fractions from trained and sedentary hearts revealed comparable values for total homogenate, myofibril (169.2 ± 2.0 (S) versus 161.0 ± 3.0 (T) $mg \cdot g^{-1}$ wet tissue), actomyosin, mitochondria and 15,000 x g supernatant protein. However, a more severe protocol elicited greater ATPase adaptation and was associated with a significant increase in heart weight (Bhan and Scheuer, 1972). Thus, moderate swimming programs have shown elevated ATPase activities without apparent selective increases in myofibril protein (Bhan and Scheuer, 2975; Malhotra et al., 1976; Giusti et al., 1978). Endurance running produced a significant elevation in myosin Ca^{2+}-ATPase activity in the absence of increased heart weight (Resink et al., 1981a) while increases in cardiac performance without altered actomyosin ATPase showed similar myofibril protein yields with treadmill-running in male rats (60.0 ± 1.2 (T) versus 59.9 ± 0.85 (S) $mg \cdot g^{-1}$) (Tibbits et al., 1978) and in dogs (Dowell et al., 1977).

The elevation of myosin and actomyosin ATPase activities with training in the absence of quantitative protein changes indicates a modification in its enzymatic properties. Since ATP-induced turbidity has been correlated with actomyosin ATPase activity and contractility (Katz, 1970) the observation of enhanced rates and extent of superprecipitation of actomyosin from swim-trained hearts further supports the increased ATPase activity (Bhan and Scheuer, 1972). It was suggested that the increased $\dot{V}_{max}$ of superprecipitation and similar optimal ATP concentrations, profile curves and Km for ATP indicated an alteration in the region of the catalytic site (Bhan and Scheuer, 1972). Further evidence that a change occurs in the head region of the myosin molecule was provided by the elevated Ca^{2+}-ATPase activity of HMM with swimming (Bhan and Scheuer, 1975).

In order to explore possible modifications to the active region of the ATPase enzyme with endurance exercise, various chemical agents and probes have been employed to study electrostatic interactions, hydrophobic bonds and functional groups such as SH_1 and SH_2. Variation of in vitro KCl concentration from 0.03 to 0.18 M elicited a 50% increase in the myosin Ca^{2+}-ATPase activity from trained hearts with little change in that of sedentary. Further increases in KCl concentration up to 0.60 M demonstrated a sustained elevation in the trained myosin Ca^{2+}-ATPase. The activating effect of KCl in the trained group was taken to represent an alteration in electrostatic forces and configuration in the region of the active site (Bhan and Scheuer, 1975). An endurance running study with dogs which failed to show increased myofibril ATPase activity demonstrated a similar response of the ATPase in trained and sedentary hearts to varying KCl where increasing KCl up to 0.20 M produced a decline in activity (Dowell et al., 1977). The different exercise protocols and particularly the different protein systems used probably account for the dissimilar findings regarding KCl effect. Chemical agents which disrupt hydrophobic bonds have also differentiated between the myosin Ca^{2+}-ATPase activities of endurance-swum and sedentary hearts. In the presence of 20% ethylene glycol the trained myosin ATPase activity revealed no activation in the presence or absence of Ca^{2+} compared to that of the sedentary hearts which increased 20%. Both groups showed a similar pattern of inhibition at higher ethylene glycol concentrations (Bhan and Scheuer, 1975). The apparent lack of effect of ethylene glycol further suggested a conformational change in the region of the active site. Increasing concentrations of the denaturing agent urea (0.5 to 3.0 M) caused comparable inhibitions of the two groups of myosin.

Further evidence of conformational change of the myosin head was also indicated by changes in the fluorescence intensity of HMM-ANS (8-anilinonapthalere sulforate). Modification of myosin ATPase activity such as through sulfhydryl-group blocking yields an increased fluorescence, therefore the 30% greater fluoresence of the myosin from swim-trained hearts was taken to indicate altered conformation (Bhan and Scheuer, 1975). The effect of 1 M urea was to preferentially increase control myosin fluorescence while with 2 M urea the fluorescence intensity of both groups was significantly elevated. The authors interpreted the fluorescence data with the denaturing agent urea as evidence that swim-training induced a conformational change, perhaps an uncoiling of the protein, near the active site. The lower urea concentration induced such a change in the control myosin while not further influencing the trained myosin whereas higher urea concentrations elicited greater denaturation in both groups (Bhan and Scheuer, 1975).

SULFHYDRYL GROUPS: The conformation and behavior of the myosin active site region appears to be influenced by the sulfhydryl or thiol (SH) groups of two cysteine residues located only nine amino acid residues apart proximal to the active site on the S-1 portion of the myosin heavy chains (Elzinga and Collins, 1977; Srivastava and Wikman-Coffelt, 1980; Scheuer and Bhan, 1979). The SH groups are divided into two classes: the SH_1 react quickly with sulfhydryl-directed agents while the SH_2 group reacts more slowly (Scheuer and Bhan, 1979). The effect of the SH-modifying agents on the SH_1 group alone is to increase the myosin Ca^{2+}-ATPase activity and to decrease the K^+-EDTA ATPase activity. Further reaction with the SH_2 groups such that both classes are modified leads to a decrease in the myosin Ca^{2+}-ATPase activity.

Actin plus Mg^{2+}-stimulated ATPase activity is also decreased when SH_1 + SH_2 are blocked (Srivastava and Wikman-Coffelt, 1980; Scheuer and Bhan, 1979). The extent of the above effects appears to depend on the particular mercurial compound used and the species of myosin (Scheuer and Bhan, 1979).

Investigation into the reactivity of the SH groups with physical training in order to understand the altered myosin Ca^{2+}-ATPase activity has employed a number of mercurial compounds. Determination of the SH group content of myosin revealed that control and trained hearts had comparable amounts of SH groups per mole myosin (Bhan et al., 1975). Selective modification of the SH_1 groups with low concentrations of NEM (N-ethylmaleimide) stimulated the control Ca^{2+}-ATPase by 20% but failed to influence the ATPase from trained hearts. Increasing NEM concentrations resulted in decreased ATPase activity in both groups. Incubation with CMS (p-chloromercuriphenyl sulfonate) strongly inhibited the Ca^{2+}-ATPase of both groups (Bhan et al., 1975). Differential effects of IAA (iodoacetamide) on control and swim-trained myosin were also reported (Scheuer et al., 1974; Bhan et al., 1975). Maximal differences in ATPase activation between control and trained groups were observed with 10 mM IAA and increasing time of titration where after 2.5 minutes the maximal ATPase of the control group exceeded that of the trained by 35%. By 10 minutes the activities were similar. The authors suggested that the biphasic pattern of response indicated that thiol groups with two different reactivities to IAA were influencing ATPase activity. The difference between trained and control activities was interpreted as a dissimilarity in the availability and reactivity of the rapidly reacting thiol groups (SH_1) (Bhan et al., 1975). Incorporation of radioactive ^{14}C-IAA into the two groups of myosin was shown to be 25%

lower in the trained group compared to controls with 2.5 minutes titration, whereas after 10 minutes titration with ^{14}C-IAA similar values between groups were observed corresponding to the similar myosin ATPase activities (Bhan et al., 1975). The presence of MgATP during a 2.5 minute titration resulted in similar extents of ^{14}C-IAA incorporation into trained and control myosin abolishing the previous difference and having a greater relative effect on incorporation into control myosin versus the trained. From these observations it was proposed that the MgATP effect indicates participation of some SH groups in nucleotide binding in the region of the active site. Moreover, the relatively lower incorporation of ^{14}C-IAA into the trained myosin versus control suggests that endurance swimming may promote a change in the substrate-binding SH groups (Bhan et al., 1975).

ISOENZYMES: As a result of various physiological and pathological cardiac overloads, the distribution of myosin isoenzymes has been observed to shift towards V_1 or V_3 dominance (Rupp, 1981; Hoh et al., 1978). Structural modification of the myosin protein may then contribute to altered mechano-chemical properties of the myofibrils (Rupp, 1981). An endurance swimming program using male Wistar rats, which elicited a 10% increase in the Ca^{2+}-activated cardiac myofibril ATPase activity, also caused a pronounced shift in isoenzyme pattern to the faster V_1 form with only small amounts of V_2 and V_3 measured (Rupp, 1981). By contrast, hemodynamic pressure-overload due to renal hypertension in another group of rats resulted in a shift towards the slower V_3 predominance (Rupp, 1981). Since cardiac myosin polymorphism arises from differences in heavy chain composition (Hoh et al., 1978), the alterations in behavior of the protein near the active site with training as previously discussed may be partially attributable to the isoenzyme characteristics.

The trigger for such adaptations remains obscure but with exercise may be related to the increased cardiac output (Rupp, 1981). The significance of different isoenzyme patterns appears to relate to the type of myocardial work required. Increased heart rates and ATP turnover in chronically-exercised hearts may require greater rates of cross-bridge cycling as permitted by V_1 dominance whereas in hemodynamic overload a slower rate of ATP hydrolysis may allow greater economy of tension development (Rupp, 1981). The structural and biocheimcal changes in myosin ATPase activity may then be further modified by regulation of Ca^{2+} at the level of the sarcolemma or intracellular stores (Rupp, 1981).

MYOFIBRIL PROTEIN PHOSPHORYLATION: Covalent modification of several muscle proteins by phosphorphylation-dephosphorylation mechanisms has been shown to regulate muscle function at least in smooth and skeletal muscle and possibly in cardiac muscle as previously discussed in Section III (Barany and Barany, 1980). In cardiac muscle cAMP-dependent phosphorylation of the serine-20 residue in TnI has so far proven to be the only clearly functionally-significant form of covalent modification (Holroyde et al., 1979a; Winegrad et al., 1983) although the poorly understood role of Ca^{2+}-calmodulin dependent cardiac myosin P-light chain regulation is being heavily investigated (Kopp and Barany, 1979; Reddy and Wyborny, 1979; Resink et al., 1981a, b) (See Section III). The possible contributions of myosin P-light chain and TnI phosphorylation and of their respective Ca^{2+}-and cAMP-dependencies to the observed adaptations in myosin and actomyosin ATPase activities accompanying endurance training have been investigated (Resink et al., 1981a, b).

Following a strenuous endurance running program, trained male rat hearts showed significantly increased myosin Ca^{2+}-ATPase activity but no differences in the myosin K^+-EDTA or Ca^{2+}-activated Mg^{2+}-stimulated actomyosin ATPase activities compared to sedentary counterparts (Resink et al., 1981a; Table A-2). Treatment of isolated perfused hearts with isoproterenol induced the expected phosphorylation of both Tn-I and myosin P-light chains (Perry, 1979). However a greater extent of alkali-labile phosphate incorporation into the P-light chains was recorded for trained hearts versus sedentary while the two groups showed similar extents of TnI phosphorylation. With regard to the catecholamine effects on TnI, there was also no difference in the degree of adenyl cyclase activation and cAMP formation in the two groups. Furthermore the cAMP-dependent phosphorylation of TnI temporally correlated with decreases in the Ca^{2+}-sensitivity and V_{max} of the actomyosin Ca^{2+}/Mg^{2+}-ATPase activity in the two groups (Resink et al., 1981a).

Isoproterenol stimulation resulted in an elevation of the myosin Ca^{2+}-ATPase V_{max}, this effect occurring to a greater extent with trained hearts which already shared higher basal V_{max} activities (Resink et al., 1981a). Increased V_{max} correlated temporally and quantitatively with greater relative phosphorylation of myosin P-light chains in the trained hearts (T: 0.38 ± 0.01 versus S: 0.26 ± 0.01 mol ^{32}P incorporated·mol P-light chain^{-1}). K^+-EDTA-ATPase activities, which reflect SH_1 and SH_2 integrity, were not influenced by catecholamine stimulation (Resink et al., 1981a).

From the above results the authors concluded that the adaptive increase in the Ca^{2+}-ATPase activity of trained hearts is mediated in part by enhanced Ca^{2+}-dependent phosphorylation of myosin P-light chains. This response is amplified upon catecholamine stimulation to a greater degree with training (Resink et al., 1981a). The elevated phosphorylation was suggested to arise from 4 possible mechanisms: 1) greater susceptibility of P-light chains to phosphorylation by the Ca^{2+}-calmodulin-dependent myosin light chain kinase (MLCK), 2) enhanced levels of MLCK, 3) decreased light chain phosphatase activity, or 4) increased Ca^{2+} availability to the MLCK system. Since Tibbits et al. (1978) demonstrated that enhanced Ca^{2+} transport from the extracellular space accompanied training the fourth alternative was the preferred explanation (Resink et al., 1981a). Subsequent work to determine further whether a Ca^{2+}-linked mechanism is partly responsible for myocardial adaptation to training revealed enhanced myosin Ca^{2+}-ATPase activities and phosphate contents of P-light chains in response to increasing Ca^{2+} concentration. Trained hearts demonstrated 10% and 30% increases in V_{max} with increases to 2.2 mM and 3.6 mM free Ca^{2+} while sedentary hearts responded only at 3.6 mM Ca^{2+} with a 20% increase in V_{max} (Resink et al., 1981b). This greater sensitivity to alterations in extracellular Ca^{2+} concentrations appears to accompany training. Isoproterenol infusion resulted in amplification of the ATPase and P-light chain phosphorylation and, when combined with increased extracellular Ca^{2+}, led to greater relative increases in the trained versus sedentary hearts. Least squares linear regression analysis of the relationship between myosin Ca^{2+}-ATPase V_{max} and P-light chain phosphorylation yielded a 0.44 correlation coefficient (Resink et al., 1981b).

In summary, with regard to myosin P-light chain involvement, changes in the extent of their phosphorylation have been directly associated with the contractile state of the myocardium (Kopp and Barany, 1979). Therefore Resink et al. (1981b) propose that augmented contractility with training, results from enhanced P-light chain phosphorylation which exert their effects through potentiation of myosin ATPase activity and cross-bridge activation. Increased Ca^{2+} transport into the intracellular space further activates the Ca^{2+}-calmodulin MLCK and subsequent phosphorylation.

The lack of involvement of cAMP in myocardial adaptation to exercise training is evidenced by the similar levels of cAMP, the similarities of the catecholamine-induced decreases in V_{max} and Ca^{2+} sensitivity of the Ca^{2+}/Mg^{2+} actomyosin ATPase activities, by the similarities in the cAMP-dependent phosphorylation of Tn-I after isoproterenol infusion and by the similarity of the response of cAMP levels to elevated levels of perfusate Ca^{2+} between sedentary and trained hearts (Resink et al., 1981[a,b]). The role of TnI phosphorylation probably resides in its enhancement of relaxation (Winegrad et al., 1983) and in its reduction in the extent of troponin-regulated thin-filament transduction possibly smoothing out the inotropic response to elevated cAMP and Ca^{2+} (Perry, 1979; Resink et al., 1981b) which is not enhanced with training. To this date, there is no evidence in the literature which clearly shows training adaptations in thin-filament regulation of myofibril ATPase activity (Resink et al., 1981b).

SARCOPLASMIC RETICULUM: The possibility that enhanced myocardial performance and ATPase activity with endurance training is attributable to improved Ca^{2+}-transport by the sarcoplasmic reticulum (SR) has received some support. Penparkgul et al. (1977) demonstrated increased Ca^{2+}-storage in microsomes, increased rate and extent of Ca^{2+}-uptake with 1 mM oxalate and a greater response to increased myoplasmic Ca^{2+} levels. A further study agreed and also showed increased Ca^{2+}-binding with no alterations in the Ca^{2+}/Mg^{2+} SR-ATPase activity (Penparkgul et al., 1979). The primary result of such adaptations was thought to involve increased rate of myocardial relaxation (Penparkgul et al., 1977).

Endurance running failed to elicit consistent and significant elevations in Ca^{2+}-binding and uptake although the initial velocity of Ca^{2+} uptake was significantly greater in run-trained hearts at 1 mM oxalate (Penparkgul et al., 1980). The apparent difference between swimming and running as regards SR function may explain the observed increases in myocardial relaxation rate with swimming (Bersohn and Scheuer, 1977) which are apparently absent with running (Schaible and Scheuer, 1979).

5. ENERGY METABOLISM

Distinct and marked increased in the energy-producing capabilities of skeletal muscle, specifically the oxidative capacity, in response to endurance training have been well-documented (Oscai et al., 1971; Holloszy, 1976). In contrast, studies with whole heart preparations and isolated subcellular biochemical systems have indicated few compensatory changes in the energy-generating capacity of cardiac muscle in response to endurance exercise (Holloszy, 1976; Sordahl et al., 1977; Oscai et al., 1971; Scheuer et al., 1974). Therefore, the improved cardiac performance with training may not be attributable to increased oxidative capacity of cardiac muscle (Scheuer and Tipton, 1977). In this regard, there is some evidence to suggest that more subtle adaptations may lead to improved efficiency of the oxidative pathway (Sordahl et al., 1977; Penparkgul et al., 1978). The parameters involved in energy metabolism include coronary flow, O_2 supply and demand, substrate supply, substrate utilization by the glycolytic and oxidative pathways, mitochondrial capacity and enzymatic profiles. The net result is ATP production for cellular energy-dependent mechanisms.

CORONARY FLOW: Studies utilizing isolated perfused hearts from endurance-swum rats have indicated that coronary flow is enhanced with training, particularly when the hearts are placed under increased stress such as at elevated preloads (Penparkgul and Scheuer, 1970; Bersohn and Scheuer, 1977; Scheuer and Tipton, 1977). However, it has been suggested that coronary flow in trained hearts may be reduced at rest and at submaximal workloads due to the lower oxygen requirements of the heart (Barnard et al., 1980). Indeed, determination of total and regional coronary blood flow measured by microsphere injection revealed small but significant reductions in flow at rest and at submaximal workloads in treadmill-trained dogs, while no differences were observed at maximal exercise. Concomitant reduction in myocardial O_2 demands as indicated by tension-time index were also observed (Barnard et al., 1980).

Increases in the size of the coronary tree has been reported with endurance training. Increased capillary to fiber ratios, increases in cross-sectional areas of extracoronary collateral arteries and in major coronary arteries and increases in the ratio of vascular space to myocardial mass have been observed in endurance-trained rat hearts (Dawson and Horvath, 1970; Scheuer and Tipton, 1977). These results have not been supported by studies involving larger animals. Barnard et al. (1980) reported no differences in the maximal coronary blood flow per gram of tissue after 12 to 18 weeks of intensive running in dogs. The coronary tree size was suggested to increase only in proportion to the increased muscle mass by observations of unaltered subendocardial vascular resistance. Similarly, Cohen et al. (1978) were unable to demonstrate a training effect on collateral vessel development in dog hearts. The discrepancies in the literature as regards coronary vascular adaptations to training may be species-related where larger hearts possibly possess greater coronary dilator reserves in response to maximal exercise (Barnard et al., 1980). The differences may also extend to methodological considerations and to the training stimuli.

OXYGEN SUPPLY AND DEMAND: Isolated, perfused trained rat hearts have exhibited elevated $m\dot{V}O_2$ (myocardial $\dot{V}O_2$) at higher workloads (Giusti et al., 1978; Bersohn and Scheuer, 1977; Schaible and Scheuer, 1979). The increased $m\dot{V}O_2$ was attributed to increased coronary flow in one study (Penparkgul and Scheuer, 1970). A proportionately greater $m\dot{V}O_2$ with increasing atrial pressures achieved by increased coronary flow rather than altered O_2 extraction in trained hearts was contrasted with lesser increases of $m\dot{V}O_2$ of sedentary hearts achieved by greater O_2 extraction and no flow adaptations. It was suggested that sedentary hearts possess a limitation in their coronary flow reserve (Scheuer et al., 1974). Increased arterio-venous (a-v) O_2 differences have also been reported with training (Bersohn and Scheuer, 1977; Schaible and Scheuer, 1979). The possible increase in myocardial O_2-extraction with training could arise from altered blood flow, altered mitochondrial function, or decreased oxy-hemoglobin affinity particularly at low pO_2 although this issue is controversial (Scheuer and Tipton, 1977). The efficiency of conversion of O_2 to external work was not significantly different between sedentary and trained hearts in one study indicating that increased myocardial work output was proportionately accompanied by increased $m\dot{V}O_2$ (Bersohn and Scheuer, 1977) while in a similar work, both swim- and run-trained hearts showed greater external efficiencies at high workloads (Schaible and Scheuer, 1979).

In apparent contradiction to data with trained rat hearts, data from dogs revealed that training resulted in reduced myocardial O_2 demands at rest and at submaximal loads. Potential subendocardial O_2

supply as determined from the diastolic pressure-time index was similarly reduced providing trained dog hearts with a more favourable myocardial O_2 supply-demand balance (Barnard et al., 1980). Training had no effect on these indices at maximal exercise.

SUBSTRATES: Glucose extraction has been shown to decrease less in trained hearts during exercise while lactate extraction was greater despite lower arterial lactate levels for any given workload. Free fatty acid (FFA) extraction was similar between sedentary and trained hearts except with prolonged exercise where FFA utilization increased (Scheuer and Tipton, 1977). Endogenous triglyceride (TG) stores were slightly decreased in trained rat hearts (Froberg, 1971; Scheuer et al., 1974) as were blood TG and FFA levels due to slightly increased FFA turnover (Scheuer and Tipton, 1977). Cardiac glycogen stores appear to be elevated in trained hearts at rest as is glycogen synthetase activity. Reduction of cardiac glycogen during exercise to a greater degree in trained hearts was followed by a marked supercompensation in the trained hearts (Scheuer and Tipton, 1977). Injection of isoproterenol also indicated greater glycogen utilization in trained hearts (Scheuer et al., 1974).

GLYCOLYSIS: Generally, no major changes in the glycolytic pathway are reported with training in heart muscle. The major glycolytic markers enzyme, phosphofructokinase (PFK) is not altered (Baldwin et al., 1975) while pyruvate kinase and lactate dehydrogenase (LDH) are increased (Scheuer and Tipton, 1977; Scheuer et al., 1974). Under steady state conditions, an increment in heart rate led to the production of greater lactate and pyruvate in sedentary hearts while trained hearts compensated by increasing their $m\dot{V}O_2$. Left atrial pressure increments also elicited greater lactate production in trained hearts (Scheuer et al., 1974).

Under hypoxic conditions, isovolumetric or working perfused hearts of the two groups showed no differences as regards lactate production, lactate:pyruvate ratios or accumulation of NADH. However, recovery of lactate values to resting levels occurred more quickly in the trained group (Scheuer and Stetzoski, 1972).

OXIDATIVE CAPACITY: Exercise studies have shown substantial increases in mitochondrial protein content associated with hypertrophy (Arcos et al., 1968), in mitochondrial protein per gram wet heart weight in the absence of hypertrophy (Penparkgul et al., 1978), or have reported no changes in mitochondrial yield (Oscai et al., 1971; Sordahl et al., 1977). Most studies have demonstrated normal mitochondrial function (Scheuer and Tipton, 1977). A large body of research utilizing either swimming or running protocols has failed to demonstrate increases in cardiac oxidative enzyme activities per milligram of mitochondrial protein including succinate dehydrogenase (SDH), cytochrome oxidase, and citrate sythase (Oscai et al., 1971; Scheuer et al., 1974; Baldwin et al., 1977; Sordahl et al., 1977; Penparkgul et al., 1978). In addition, cytochrome C concentration also remained unchanged (Oscai et al., 1971). No differences in the ADP:O ratio (phosphorylation efficiency), or in the RCI (respiratory control index = ratio of the rate of O_2 consumption in the presence of ADP to that in the absence of ADP) were reported for run-trained dog and swim-trained rat hearts with either succinate or the NADH-linked intermediates glutamate-malate and pyruvate-malate as substrates (Sordahl et al., 1977; Penparkgul et al., 1978). State 3 O_2 uptake in mitochondria from conditioned hearts was depressed by 15% and slightly lower in State 4, but due to the increased total mitochondrial content, overall O_2 uptake per gram of heart was not reduced (Penparkgul et al., 1978). The unchanged ADP:O ratio and RCI in the face of reduced mitochondrial respiratory activity indicated that oxidative phosphorylation by mitochondria are very tightly coupled in conditioned hearts and that the efficiency of ATP production is well-preserved (Penparkgul et al., 1978). Levels of high energy phosphates in trained and sedentary hearts have been found not to differ (Scheuer and Tipton, 1977).

Evidence suggests that the energy-utilizing rather than the energy-generating processes in cardiac muscle undergo adaptation to the training stimulus. In this regard, myofibril protein and sarcoplasmic reticulum energy-dependent mechanisms have already been discussed. In addition, alterations in mitochondrial Ca^{2+} transport have been reported (Sordahl et al., 1977; Penparkgul et al., 1978). Calcium uptake per milligram mitochondrial protein was depressed 25% in conditioned hearts when respiratory substrates (glutamate and malate or succinate) and 1 to 10 mM MgATP or 3-10 mM MgADP were present while energy-independent and respiratory-supported Ca^{2+} uptake in the absence of ATP or ADP showed no differences (Penparkgul et al., 1978). Inhibition of the mitochondrial rate of Ca^{2+} uptake with ruthenuim red revealed fewer binding and/or transport sites in mitochondrial membranes from exercise-trained hearts (Sordahl et al., 1977). An inability of mitochondria to retain accumulated Ca^{2+} was also observed in one study (Sordahl et al., 1977) but not in another (Penparkgul et al., 1978). The significance of these mitochondrial Ca^{2+}-metabolism alterations remains unclear.

V. DIABETES

Classification

Diabetes mellitus is now recognized to be a highly heterogenous condition which encompasses widely variable pathogenic patterns and manifestations. In order to more precisely characterize diabetic syndromes a three-part classification scheme has beenddescribed by Brown et al. (1983). Briefly the categories are as follows:

Type I : Insulin-dependent diabetes mellitus (IDDM)

- a) also referred to as Juvenile onset diabetes (JOD).
- b) dependent on insulin to prevent ketosis or to preserve life.
- c) low plasma insulin levels.
- d) associated with certain HLA (histo-compatibility allele) systems.
- e) associated with autoimmune phenomena, especially islet cell antibodies.

Type II : Non-insulin dependent diabetes mellitus (NIDDM)

- a) previously maturity-onset diabetes (MOD).
- b) not ketosis prone but may require some insulin to control hyperglycemia.
- c) no HLA association.
- d) no autoimmune phenomena.
- e) frequently associated with obesity and adult-onset.

Type III: Secondary diabetes

- a) hormonal abnormalities.
- b) drugs and chemical agents.

c) pancreatic disease.
d) insulin receptor abnormalities.
e) genetic syndromes.

Etiology of Idiopathic Diabetes

Current thinking regarding the etiology of IDDM diabetes favours the hypothesis that genetic predisposition may be conferred by at least two genes in chromosome 6. The reaction of such predisposed individuals to certain environmental stimuli (β-cell cytotoxic virus or chemicals) is abnormal and leads to β-cell destruction directly through autoimmune mechanisms or due to lack of β-cell regeneration after damage (Nerup, 1981). This theory, though based on a growing body of literature, remains highly speculative.

A. Genetics:

The idea of autosomal recessive inheritance of diabetes is no longer considered acceptable; rather, on this basis a multifactorial inheritance pattern appears more logical. However a special form of NIDDM that occurs in youth is associated with mild hyperglycemia, a strong family history and therefore may be dominantly inherited.

Most genetic research is focused on the association of specific alleles of the HLA system on chromosome 6 with increased risk of developing IDDM. D locus alleles appear more closely linked to a putative diabetic gene than other implicated loci (A and B). Additionally, certain alleles may exert a protective effect.

B. Immunology:

Islet Cell Antibodies: Islet cell antibodies are prevalent in newly-diagnosed IDDM patients but do not appear strongly related to disease-duration. Persistent islet cell antibodies have an increased association with specific alleles in the histocompatibility system and are considered to cause diabetes of an autoimmune origin. Rapid clearance of plasma antibodies to islet cells suggests an environmental origin.

Lymphocyte function may also be abnormal in diabetes.

C. Viruses:

A number of different viruses are known to cause diabetes in experimental animals or man. Susceptibility to diabetes resulting from viral infection however is not uniform and may be influenced at several levels. Host susceptibility to the virus may be genetically-regulated, viral strains may vary in pathogenic capabilities and the susceptibility of pancreatic viral infection may also be under genetic control. The HLA characteristics of the individual will also determine antibody capacity. At present human evidence of these mechanisms extends only to case reports.

D. Abnormal Insulin:

A defect in insulin structure and/or the biological activity of the hormone provide current explanations of NIDDM. The five main causes are:

1) pre proinsulin structural gene mutation.
2) incomplete conversion of proinsulin to insulin.
3) circulating antagonists to insulin.
4) defects in the insulin receptor site.
5) defects in target cell responsiveness at a site distal to hormone binding.

(Brown et al., 1983).

Pathogenesis of Diabetes

In controlled diabetes in man, the following pathophysiological changes have been reported (Brown et al., 1983):

Pancreas: In the Islets of Langerhans β-cell necrosis, glycogen infiltration of β-cells, fibrosis, hyalinization and lymphocytic infiltration may occur to some extent during various stages of the disease. Insulin secretion is impaired or abolished.

Cardiovascular System: Incidence of atherosclerosis is elevated and occurs at an earlier age in diabetics. It accounts for 75%-80% of diabetic deaths. Large vessels more severely affected are those of the coronary, peripheral and renal circulation. Alterations in small vessels and microangiopathy also occur more frequently in diabetics. Microangiopathy occurs most commonly in skin, muscle, peripheral nerves, eyes and kidneys.

Kidneys: The kidneys are affected at several levels of tissue function and structure. Vascular changes, glomerulosclerosis, diffuse or nodular glomeruli, fibrosis or hyalinization of the Bowman's Capsule, vacuolization of tubules, pyelonephritis and renal papillary necrosis all severely disrupt kidney function.

Eyes: Microvascular changes result in retinopathy.

Brain: Cerebrovascular impairments, dorsal column degeneration and peripheral neuropathy.

Liver: Fatty infiltration.

Skin: Vascular and neuropathic alterations

Muscle: Structural and functional degeneration.

Experimental Models of Diabetes

The purpose of developing animal models is to use them to elucidate problems of human pathology which cannot be solved in clinical experiments. As regards diabetes, numerous spontaneously-diabetic and experimentally-induced models have been employed but display diverse pathophysiology such that no one model precisely represents a type of human diabetes. However, in view of the large diversity that characterizes human diabetes, animal models collectively provide an extremely useful means for understanding the disease process (Mordes and Rossini, 1981).

Spontaneous diabetes is best understood in rodents although it occurs in many species. Hyperglycemia and at lease transient hyperinsulinemia characterize the spontaneous diabetic syndrome in animals, most of which are obese. The lean BB rat, a mutation of a Wistar population, displays many characteristics of human diabetes including hyperglycemia, hypoinsulinemia, some ketoacidosis, elevated counter-regulatory hormones, pancreatic insulinitis, reduced β-cell numbers and lymphocytic and macrophagic infiltration. This model is thought to represent an example of cell-mediated immune pathogenesis. Other spontaneous diabetes models include the Chinese hamster, South African hamsters, varieties of an obese mouse, the mildy-obese Japanese KK mouse and varieties of animals where diabetes is related to dietary stress. In the obese models, hyperglycemia, hyperinsulinemia and insulin resistance are characteristic although their metabolic relationship to non-insulin-dependent diabetes is uncertain (Mordes and Rossini, 1981).

Experimentally-induced diabetes may be the result of surgery, viral infection or administration of various hormones and chemical-agents. Pancreatectomy, administration of contra-insulin hormones, electrolytic or chemical hypothalamic lesions, species-specific RNA viruses and various toxic chemicals offer a broad spectrum of experimental procedures to the researcher (Mordes and Rossini, 1981).

Toxic diabetes is especially useful in that it permits biochemical, hormonal and morphologic study throughout the course of the disease. Cell-specific toxins which destroy β-cells and cause a primary insulin deficiency, agents which act on the β-cells without destruction and finally, agents which increase endogenous insulin requirements and indirectly produce diabetes comprise the three classes of toxic agents. Allozan and streptozotocin are the two primary cheimcal agents in experimental diabetes. Both are β-cell cytotoxins which, in diabetogeneic doses, are relatively free of nonspecific toxic effects (Mordes and Rossini, 1981).

The mechanism of alloxan's (mesoxalylurea) cytotoxic effect is not known, however the initial step may involve some form of membrane action on the surface of the β-cell. Alloxan demonstrates a high affinity for islet cell membranes and may possibly effect membrane permeability changes resulting in necrosis (Mordes and Rossini, 1981). Although considered a specific toxin, alloxan has been reported to produce lesions in organs such as kidneys, liver and various nonpancreatic endocrine tissues during the first weeks of administration as well as interfering with intestinal transport in the rat (Goodman and Hazelwood, 1971).

Streptozotocin, an N-nitroso derivative of D-glucosamine, was discovered after alloxan and was originally a metabolite of streptomyces achromogenes, though now synthetic varieties are more available. Maximal stability of the drug is obtained at an acidic pH of 4.5 (Wiley, 1981). Streptozotocin (STZ) is a broad-spectrum antibiotic that possesses 4 main biological properties represented by its antibiotic, oncogenic, oncolytic and β-cytotoxic effects (Like and Rossini, 1976; Ganda et al., 1976). The first step of the β-cytotoxic effect of STZ probably involves cell membrane binding. Specific recognition by a receptor on on the β-cell is suggested by the greater cytotoxic effect of the α anomer of the glucosamine moiety compared to the β anomer (Mordes and Rossini, 1981). The glucose component of the molecule may also enhance its uptake into the β-cell. Within the β-cell, STZ is thought to decrease NAD content by decreasing its synthesis and its degradation. Nicotinamide injection prior to STZ or alloxan injection prevents the development of diabetes (Mordes and Rossini, 1981; Junod et al., 1969) although alloxan has not been shown to influence β-cell NAD levels (Ganda et al., 1976). STZ, as alloxan, is most commonly given as a single diabetogenic injection. After STZ clearance from the bloodstream, serum half-life is 15 minutes and light microscope evidence of β-cell necrosis appears within 24 hours which can be ultrastructurally-detected within 2-4 hours (Like and Rossini, 1976; Junod et al., 1969). Intramembranous particle depletion of β-cell plasma membranes is observed within 45 minutes. Dissolution and phagocytosis of nocrotic β-cells occurs rapidly with little evidence of inflammation by 3 days (Like and Rossini, 1976). These histopathologic developments correspond to development of hyperglycemia (Junod et al., 1969).

Examination of the interaction of several carbohydrate compounds on the β-cytotoxic effects of STZ and alloxan has lent some insight into mechanisms of cytotoxic actions and differences or similarities between the two agents (Ganda et al., 1976). Protection against alloxan toxicity is afforded by prior injection of D-glucose, D-mannose and 3-O-methyl-D-glucose with removal of the protection by D-mannoheptulose. As regards STZ, only 3-O-methyl-D-glucose administered immediately prior to STZ injection prevented β-cell toxicity in a concentration-dependent manner. Mannoheptulose did not alter the protective effect. In addition 2-deoxy-D-glucose, which has no effect on alloxan, provided protection against STZ though to a lesser degree. The effects of these two compounds were additive in preventing β-cell destruction when injected together. This evidence strongly suggests that STZ and alloxan interact at different sites on the β-cell membrane. That a cell-surface site is involved is indicated by the protective effect of 3-O-methyl-D-glucose, a nonmetabolized analogue of glucose which cannot stimulate insulin secretion. Lack of protection by glucose and the ineffectiveness of mannoheptolose with STZ as compared to alloxan support different binding sites as well (Ganda et al., 1976).

Despite possible differences in mechanism of action on β-cells, STZ and alloxan exhibited a similar hyperglycemic response when given in comparable doses until the level of 60-70 mg/kg where higher doses of alloxan elicited a greater hyperglycemic response (Ganda et al., 1976).

Development of the acutely diabetic state also seems to follow a similar course with STZ and alloxan. A triphasic response following injection has been observed in some parameters. Blood glucose, FFA and TG levels increased up to about 12 hours post-injection, returned to near base-line at 18 hours, then increased until 48 hours after alloxan (60 mg/kg) administration. The insulin response corresponded inversely to these parameters (Meier et al., 1972). In this same study accelerated ketogenesis appeared at 24-30 hours and reached peak levels (17-18 mmol/dl) at 48 hours. The pattern of glucose response following a 65 mg/kg dose of STZ was similar although compressed into an earlier timeframe where elevations at 2 and 4 hours were followed by a sharp drop at 7 hours and a continuing rise from 10 to 24 hours. An inverse insulin pattern was again observed to correspond with that of the glucose response (Junod et al., 1969). In contrast to the alloxan study, a similar dose of STZ did not elicit any measurable ketonuria; Ketosis was however induced with a much higher STZ dose (100 mg/kg). Metabolic stability was observed between 7 and 28 days. The best index of the STZ diabetogenic effect was regarded as being pancreatic imunoreactive insulin (IRI) levels after 24 hours. Graded states of overall pancreatic insulin depletion were produced by varying STZ doses from 25 to 100 mg/kg. The authors concluded that STZ differed from alloxan by 4 main characteristics:

1) the selectivity of STZ-action on β-cells is greater,
2) the relationship between dose and diabetogenic response differs with STZ having a broader range,
3) subdiabetogenic doses of STZ leads to increased sensitivity to a second dose, and
4) the diabetogenic action of STZ is only mildly affected by nutritional state whereas major changes have been reported for alloxan effect in the fasted or fed state.

(Junod et al., 1969)

It may therefore be suggested that STZ is the preferred β-cytotoxic agent.

Metabolic and Functional Alterations in the Diabetic Heart

Primary abnormalities in energy metabolism in diabetes mellitus (IDDM) arise from a deficiency or absence of insulin. This peptide hormone, produced by the pancreatic β-cells in the Islets of Langerhans, is the major anabolic or energy storage hormone in mammals with multiple effector sites such as skeletal and cardiac muscle, adipose tissue and liver. In muscle, insulin facilitates glucose uptake and utilization and promotes glycogen synthesis, amino acid uptake and protein synthesis. Its influence on adipose tissues involves restriction of free fatty acid (FFA) and glycerol release through inhibition of hormone-sensitive lipase, increased glucose uptake and increased triacylglycerolrsynthesis. The prime insulin target is the liver where insulin action elevates glycogen synthesis, and depresses gluconeogenesis and ketogenesis. Secondary effects of insulin lack usually involve further alterations in components of biochemical pathways directly influenced by insulin (May, 1981).

In addition to the primary role of insulin deficiency in diabetes, a role in the pathogenesis of hyperglycemia and other metabolic disturbances of diabetes has been attributed to insulin counterregulatory hormones which, under some conditions, are elevated in diabetes (Gerich et al., 1981). The major counterregulatory hormone is glucagon, a peptide hormone produced in the a-cells of the pancreatic Islets of Langerhans. Its major effector site is the liver where it increases hepatic glycogenolysis, gluconeogenesis and decreases hepatic glycolysis. The glucagon:insulin ratio is physiologically-important where an increased ratio elicits energy mobilization from the liver but is highly sensitive to very small alterations in insulin levels (May, 1981). Glucagon also has positive ionotropic effects increasing contractile force, cardiac output and arterial pressure. The other counterregulatory hormones include catecholamines, cortisol and growth hormone which all exert major catabolic effects on hepatic and/or peripheral glucose metabolism (Gerich et al., 1981).

In the absence of adequate insulin, and in the presence of increased levels of counterregulatory hormones, abnormalities in carbohydrate, lipid and protein metabolism develop and may lead to death as a consequence of metabolic acidosis and severe dehydration.

Metabolic disturbances in diabetes are widespread, however this discussion will focus only on those known to occur in the myocardium. In the adult heart, energy to support mechanical activity is derived almost completely from aerobic metabolism via oxidative phosphorylation in the mitochondria. In the fed state, glucose is the major metabolic fuel since blood glucose and insulin levels are high, and plasma FFA concentrations are low due to decreased mobilization from adipose tissue, while under conditions of increased metabolic demand the myocardium relies more heavily upon fatty acid oxidation. In diabetes, the myocardial metabolism is based preferentially towards these lipid pathways as plasma levels of FFA and ketones are high, with a subsequent impairment of glycolysis and pyruvate oxidation and an accumulation of products of incomplete fat combustion. Thus in the heart, rates of glycolysis are controlled by plasma concentrations of glucose, FFA, ketones, by cardiac cell lipolysis as well as by the levels of circulating hormones such as insulin and catecholamines (Wood et al., 1981; Sinclair-Smith, 1979).

Catabolism of nutrient molecules such as glucose or FFA in the heart provide carbon skeletons to the tricarboxylic acid cycle (TCA) which, with the electron transport chain, generate ATP by the process of oxidative phosphorylation. Hydrolysis of ATP by contractile action of the heart generates ADP which stimulates increased oxygen utilization and resynthesis of ATP by the mitochondrial electron transport chain. It is evident then that abnormalities in the catabolism of the nutrient molecules may influence ATP generation and thereby alter myocardial function as has been observed in diabetes (Opie et al., 1979; Miller, 1979; Wood et al., 1981; Neely and Morgan, 1981).

Research examining the metabolic abnormalities in the diabetic myocardium has relied heavily upon the experimental diabetes model where diabetes is induced by injection of alloxan or streptozotocin. Following acute or chronic progression of the disease, biochemical tissue analyses and/or the response of isolated perfused heart models to various substrates, to increased heart work or to anoxic or ischemic conditions are compared to normal hearts. The experimental models elicit similar diabetogenic symptoms such as hyperglycemia, hypoinsulinemia, glucosuria and ketonuria as seen in humans, which allows some extrapolation of the research findings to the human disease. However, it is important to note the dose and duration of the experimental diabetes as there appear to be some metabolic distinctions between the acute and chronic diabetic states.

Carbohydrate Metabolism

A carrier-mediated transport of sugar down a concentration gradient across the cell membrane has been shown for heart muscle which possesses the characteristics of saturation kinetics, stereospecificity and competition for binding sites. Binding of insulin to the membrane alters the rate of glucose movement across the membrane by a mechanism as yet unknown (Neely et al., 1972; Neely and Morgan, 1981). In diabetic hearts, transport is inhibited due to deficient insulin (Opie et al., 1971; Neely and Morgan, 1981). As well, glucose transport is less sensitive to insulin stimulation as observed by the inability of insulin-stimulation to restore glucose transport in acutely diabetic hearts to levels seen in insulin-stimulated control hearts using the isolated perfused working heart model (D:9.6 ± 1.0 mg/g versus C:15.4 ± 0.8 mg/g) (Miller, 1979). In normal hearts, glucose transport is inhibited by oxidation of fatty acids and the decreased sensitivity to insulin in diabetic hearts has been associated with increased levels of intracellular fatty acids

(Neely et al., 1972).

Glucose phosphorylation also appears to be a rate-limiting step in glucose metabolism for the diabetic heart more so than in normal hearts. Glucose uptake in the presence of insulin is limited by the rate of glucose phosphorylation to glucose-6-phosphate (G-6-P) catalyzed by hexokinase (Neely and Morgan, 1981; Opie et al., 1979; Miller, 1979). Insulin-stimulation of glucose-perfused hearts increased G-6-P levels of normal hearts to a much greater extent than in acutely alloxan-diabetic heart even though 'basal' levels were similar (Miller, 1979). In streptozotocin-diabetic hearts (65 mg/kg) a significant decrease in Types I and II isozymes of hexokinase was reported as well an increase in the tissue content of G-6-P; both effects were normalized by insulin (Das, 1973). Decreased levels of hexosemonophosphates were reported for both acute alloxan- and chronic streptozotocin (STZ) - diabetic perfused hearts when glycolytic flux was increased by insulin-stimulation (Opie et al., 1971) while in chronic STZ-diabetes it has further been reported that the perfused heart has normal or decreased contents of hexosemonophates (Opie et al., 1979). Although the literature appears inconclusive regarding the role of G-6-P and hexokinase influence, it seems that the inhibition of hexokinase is secondary to accumulation of G-6-P which may increase in the face of depressed phosphofructokinase (PFK) activity reported to occur in diabetes (Neely and Morgan, 1981; Opie et al., 1979).

The enzyme PFK represents a major regulatory site of glycolytic flux (Stryer, 1981). The enzyme is allosterically regulated by several intermediates and products of glycolysis and oxidative phosphorylation: ATP (-), citrate (-), fructose-1, 6-diphosphate (F-1, 6-dP)(+), AMP (+), and Pi (+) (Wood et al., 1981). In acute, chemical diabetes PFK activity was depressed (Opie et al., 1971) while in the chronically diabetic rat heart, glycolysis was not limited at the level of PFK (Opie et al., 1979). Inhibition of PFK in diabetes is predominantly due to the elevated levels of citrate arising from an increased oxidation of fatty acids and increased lipid accumulation in diabetic cardiac tissue (Opie et al., 1971; Wood et al., 1981). Thus, elevated lipid metabolism in diabetic heart exerts a direct inhibitory effect on glycolysis at the level of PFK under some conditions. In the diabetic heart, alterations of adenine nucleotides or inorganic phosphate (Pi) have little effect on PFK activity.

The remaining restriction on glucose oxidation to CO_2 is at the level of conversion of pyruvate to acetyl-CoA, a reaction catalyzed by the multienzyme complex pyruvate dehydrogenase (PDH). This is a mitochondrial enzyme and is in direct competition with β-oxidation of fatty acids for CoASH and NAD (Neely and Morgan, 1981). Pyruvate oxidation by heart mitochondria is substantially inhibited in diabetes and pyruvate accumulation results in part due to the elevated oxidation of fatty acids and ketone bodies (Kerbey et al., 1977; Wood et al., 1981; Opie et al., 1979). PDH has therefore received much attention as a regulator of pyruvate oxidation. The PDH complex consists of 3 enzymes involved in pyruvate metabolism plus two regulatory enzymes, PDH kinase and PDH phosphatase, which control PDH activity via the phospho-dephospho mechanism where phosphorylation by the kinase is inhibitory and dephosphorylation by the phosphatase is activating (Stryer, 1981). The proportion of active PDH in rat heart mitochondria is regulated by ATP/ADP, $NADH/NAD^+$ and acetyl-CoA/CoA ratios with activity decreased especially by high $NADH/NAD^+$ and acetyl CoA/CoA ratios (Kerbey et al., 1977; Neely and Morgan, 1981). In diabetes, the amount of active PDH is decreased to 1-6% compared to 16-20% in normal heart and this effect has been associated with the higher tissue levels of acetyl-CoA and NADH which accrue from elevated lipid metabolism (Opie et al., 1979; Neely and Morgan, 1981). During contraction the proportion of active PDH increases to 41% in control hearts but only to 13% in diabetic hearts (Wood et al., 1981). However, one study demonstrated that in mitochondria from diabetic (Alloxan) and non-diabetic hearts, the phosphorylation and inactivation of PDH was enhanced by some factor other than concentration ratios of NADH/NAD$^+$, acetyl-CoA/CoA or ATP/ADP in diabetes (Kerbey et al., 1977). Impairment of PDH dephosphorylation by the phosphatase may be a factor. In addition, pyruvate inhibition of the PDH kinase seen in normal hearts is severely impaired in diabetes and is also moderately impaired by fatty acid and ketone oxidation (Wood et al., 1981). In summary, restriction of glycolytic flux at PDH may be an example of a secondary alteration in glucose metabolism resulting from elevated fatty acid mobilization in the periphery and increased myocardial dependence on fatty acids and ketones as oxidative substrates (Neely and Morgan, 1981).

Glycogen synthesis in the heart is facilitated by insulin due to increased glucose transport and increased activity of enzymes in the synthetic pathway (Das, 1973; Neely and Morgan, 1981). Synthesis is controlled by the activity of glycogen synthase which exists in a less active phosphorylated (D) form and in a dephosphorylated active (I) form. Insulin promotes phosphatase activity with dephosphorylation of the D form to the more active I synthase while G-6-P presence stimulates the D form. In diabetes glycogen synthase phosphorylase activity is significantly depressed while major intermediates of the synthetic pathway such as G-6-P, G-1-P and UDP-glucose are elevated (Das, 1973). Diabetes also results in increased myocardial glycogen which appears in direct contrast to enzyme data. However, in diabetes the glycogen synthase D form may predominate which in normal heart would repress synthetic activity but elevated G-6-P levels due to decreased PFK activity may activate the glycogen synthase D form and promote glycogen synthesis (Neely and Morgan, 1981). Thus, an alteration in the phospho-dephospho regulation of this pathway accompanies diabetes.

The presence of high fatty acid levels in the diabetic heart suggests restricted glycogenolysis although the contribution of glycogen to myocardial metabolism is uncertain (Wood et al., 1981). The degradative pathway for glycogen is also regulated by a cAMP-dependent phospho-dephospho mechanism. Catecholamine binding to membrane β-receptors elicits cAMP formation and involves Ca^{2+} and activation of protein kinases, the net result being an amplified effect converting inactive phosphorylase b to active phosphorylase a and the start of glycogen breakdown. Phosphorylase phosphatase dephosphorylates and inactivates the a form (Wood et al., 1981; Stryer et al., 1981). The influence of diabetes on the glycogenolytic cascade is not well understood. Two recent studies have indicated that experimentally-induced diabetes (perfused heart model) leads to a decrease in the ability of myocardial tissue to increase total cAMP in response to β-adrenergic receptor stimulation with isoproterenol or epinephrine, which is also reflected by a decrease in the subsequent activation of protein kinase by cAMP (Ingebretsen et al., 1981; Miller et al., 1981). The diminished cAMP response is not due to decreased basal levels of cAMP or protein kinase activity but may be due to the reduced number of β-adrenergic receptors observed in chronic STZ-diabetes (Heyliger et al., 1982). Despite diminished cAMP response, the amplification cascade system is altered in some way in diabetes such that hypersensitivity of phosphorylase activation with epinephrine and isoproterenol has been reported. It has been suggested that this effect resides in a hypersensitivity of phosphorylase kinase activation by some factor beyond protein kinase and in fact may involve altered Ca^{2+}-sensitivity of the cascade (Miller et al., 1981; Ingebretsen et al., 1981). Thus, from this research it appears that glycogencolysis in diabetic heart may occur to a greater extent than previously considered.

Lipid Metabolism

In the aerobic myocardium (not post-prandiol) FFA will be used preferentially as an energy substrate due to a greater inhibition of the glycolytic pathway. FFA uptake is a non-energy dependent process occurring by diffusion down a concentration gradient (Neely and Morgan, 1981). Elevated plasma levels of FFA in diabetes may lead to increased FFA uptake (Opie et al., 1979). In acutely alloxan-diabetic perfused rat heart, uptake of palmitate-1-C^{14} and its oxidation were reduced relative to control hearts and were associated with a two-fold increase in glycerol release from the diabetic heart. The net uptake of FFA by diabetic hearts therefore was limited by the turnover and utilization of endogenous lipids and in the perfused heart model was also related to triacylglycerol concentration in the perfusate (Kriesberg, 1966). Increased intracellular lipid turnover in diabetic hearts in the chronic state would place an increased demand on exogenous FFA utilization (Opie et al., 1979).

Cellular metabolism of FFA involves first activation to fatty acyl CoA ester (FACoA) which occurs in the cytosol. Activated FACoA can then be metabolized via a number of pathways including esterification to neutral and phospholipids, acyl chain elongation and β-oxidation via the mitochondrial electron transport chain (Wood et al., 1981; Murthy and Shipp, 1977). Myocardial triglyceride (TG) is increased in diabetes (Opie et al., 1979; Murthy and Shipp, 1977). Synthesis may proceed by 3 pathways though the major one involves transfer of the acyl units from FACoA to a-glycerol phosphate. Extent of TG synthesis in the diabetic heart also appears dependent upon the degree of diabetes. Three doses of STZ streptozotocin; 60, 85 and 110 mg/kg) elicited no increase in TG in non-ketotic hearts removed from insulin treatment, a transient increase in TG in non-insulin treated, non-ketotic rats and a stable three-fold increase in TG in severely ketotic rats respectively. This large increase was attributed to increased elevated di- and tri-glyceride formation via a pathway other than esterification of a-glycerol phosphate (Murthy and Shipp, 1977). It was also proposed that ketones participate in TG synthesis and that the data suggested an overall increased rate of endogenous TG turnover (Murthy and Shipp, 1977) which has been previously suggested by Kreisberg (1966). In non-ketotic diabetic hearts, the reduced levels of a-glycerol phosphate from glycolysis would limit TG synthesis (Neely and Morgan, 1981).

β-oxidation is favoured in the myocardium over TG synthesis since acyl groups are preferentially transferred to the abundant carrier molecule carnitine in the cytosol and directed to mitrochondrial oxidation maintaining FACoA at a relatively low cytosolic level. Fatty acids greater than 10 carbons in length require activation by an acyl-CoA synthetase followed by esterification to carnitine (as in all fatty acids) mediated by carnitine acyl transforase. The fatty acylcarnitine crosses the inner mitochondrial membrane in exchange for free carnitine followed by retransfer of the acyl unit back to CoA in the mitochondrial matrix. The acyl-CoA produced is oxidized to 2-carbon units of acetyl-CoA via β-oxidation; this step is totally dependent upon carnitine (Neely and Morgan et al., 1981; Wood et al., 1981).

Competition for acyl-CoA by carnitine acyltransferase and glycerophosphate acyltransferase (TG synthesis) is influenced by diabetes where carnitine acyltransferase activity increased 50-70% in liver although no such dependence was demonstrated in the myocardium (Wood et al., 1981). Alloxan diabetes has been shown to increase concentrations of long-chain acylcarnitine in hearts from both sheep and rats (Snoswell and Kau, 1972). Generally, in both normal and diabetic heart, regulation of fatty acid metabolism depends on availability of FFA to the acyl-CoA synthetase and carnitine acyltransferase enzymes with glycerophosphate acyltransferase exerting a lesser effect (Wood et al., 1981).

Protein Metabolism

Steady-state levels of cellular proteins are a function of synthetic and degradative pathways although altered protein content may arise from a change in only one component or both. Disturbances in this balance have been reported for hormonal deficiency and by decreased availability of oxidative substrates and amino acids (Rannels et al., 1970; Neely and Morgan, 1981). Cardiac protein synthesis is stimulated by elevated levels of amino acid substrates, by exogenous insulin, free fatty acids (Jefferson et al., 1974) and by epinephrine (Wood et al., 1981).

Diabetes is characterized by increased muscle wasting. Both heart weights (HW) and body weights (BW) of alloxan- and streptozotocin-diabetic animals were significantly decreased compared to controls while little or no change was reported in the HW:BW ratio (Neely and Morgan, 1981; Marchesini et al., 1982; Rannels et al., 1970; Griffin and Wildenthal, 1978). This negative nitrogen balance associated with diabetes appears to be chiefly a result of the loss of insulin's stimulatory effect on protein synthesis (Pain and Garlick, 1974). However, the relative contributions of decreased synthetic and increased degradative processes to diabetic protein tissue atrophy are not yet identified.

Activation of amino acids to aminoacyl derivatives by combination with transfer RNA initiates entry into the ribosome cycle where polymerization of these activated amino acids is catalyzed by a series of ribosome-dependent reactions. Peptide chain elongation from a ribosomal subunit pool, chain elongation and chain-termination constitute the cycle (Morgan et al., 1974). The rate of protein synthesis reflects the capacity or number of ribosomal subunits and levels of other enzymatic components as well as the pathway efficiency which denotes the catalytic activity of the components (Neely and Morgan, 1981). An inability of STZ-diabetic hearts to sustain normal levels of protein synthesis was attributed to both reduced capacity and efficiency (Pain and Garlick, 1974). Decreased peptide-chain initiation occurs in diabetic hearts while insulin provision removes this block which does not appear to be related to tissue levels of mRNA (Neely and Morgan, 1981). Polysome numbers are maintained in alloxan-diabetic hearts which suggests that chain initiation remains relatively rapid compared to chain elongation unlike skeletal muscle where decreased polysomes are observed (Rannels et al., 1970). Free fatty acids in heart muscle appear to maintain chain initiation in spite of deficient insulin (Morgan et al., 1974; Neely and Morgan, 1981). Peptide-chain initiation is also accelerated by high plasma levels of β-hydroxyburtarate and acetoacetate (ketones) as well as by leucine. Thus, it appears that while protein synthesis is compromised in diabetes, the myocardium is able to compensate to some extent.

Insulin, ketone bodies and leucine tend to inhibit protein degradation; the greatest effect is exerted by insulin (Neely and Morgan, 1981). Elevated rates of nitrogen excretion from human and animal diabetics suggest that diabetes enhances protein degradation (Marchesini et al., 1982). Addition of ketones and leucine to the glucose perfusate of diabetic hearts did not restore the degradative-synthetic rates to normal further demonstrating increased proteolysis in diabetic hearts (Neely and Morgan, 1981). A number of lysosomal and non-lysosomal proteases have been implicated in the degradation of endogenous proteins. Insulin lack appears to permit greater activity of some lysosomal enzymes such as cathepsin D and β-acetyl-glucosaminidase (Neely and Morgan, 1981; Dahlmann and Reinauer, 1981). Several non-lysosomal, alkaline proteases also contribute to protein breakdown. Of particular significance is the elevated excretion of 3-methyl histidine, a specific marker for myosin and actin degradation, in diabetes which implies degradation of contractile proteins possibly disrupting normal contractile function (Marchesini et al., 1982; Nagasawa et al., 1982; Dahlmann and Reinauer, 1981). A serine-type protease found in the myofibrillar protein fraction (MAP) of hearts was shown to be elevated in the hearts of myopathic hamsters (Bhan et al., 1978) and in streptozotocin-diabetic rats (Griffin and Wildenthal, 1978). More recently however it has been suggested that MAP and other group-specific proteases may be mast cell components and therefore their identification and in vitro effects arise from mast cell contamination of tissue homogenates (De Martino, 1983). Thus, MAP's contribution to in vivo regulation of protein degradation is questionable. The elevated 3-methyl histidine excretion and elevated protein degradation in diabetes still indicate increased protease activity. Candidates for this role are two neutral-alkaline proteases isolated in rat heart cytoplasmic extracts and cardiac myocytes free of mast cell contamination: 1) a Ca^{2+}-dependent protease found in many cells including mast cells and 2) an SH-dependent protease of molecular weight 25,000 d (Beinlich et al., 1982). An additional much larger SH-dependent protease (500,000 d) which degrades globin and casein to acid-soluble peptides at an alkaline pH has also been identified in cardiac, mast cell-free preparations (De Martino, 1983). The ability of insulin to reduce 3-methyl histidine excretion to normal in humans and animals (Dahlmann and Reinauer, 1981; Marchesini et al., 1982) suggests the possibility of an interaction between insulin and one or many of the possible proteases involved in protein and specifically, myofibril degradation.

High-Energy Phosphates

Several studies have documented decreases in the high-energy phosphate compounds, ATP and CP, in the hearts of diabetic animals (Opie et al., 1971; Allison et al., 1976; Opie et al., 1979; Miller, 1979; Pieper et al., 1983). In an isolated working heart model perfused with 5 mM glucose, acute alloxan-diabetes invoked a 20% decrease in ATP content which was associated with a concomitant reduction in peak systolic left ventricular pressure development and cardiac output. Insulin administration normalized ATP levels and function (Miller, 1979). Similarly, after 70 minutes of perfusion with glucose, alloxan-diabetic hearts demonstrated decreased ATP levels while ADP and AMP only transiently increased (Opie et al., 1971). In acute ketotic alloxan-diabetes, myocardial ATP and CP were decreased by 45% and 58% respectively while plasma glucose, FFA and TG were elevated; these values were normalized by insulin. Three minutes of in vitro perfusion with a 5 mM glucose, well-oxygenated buffer accelerated ATP and CP synthesis in diabetic hearts indicating a role of oxygen delivery in defective high-energy phosphate production (Allison et al., 1976). Hearts made diabetic with STZ (65 mg/kg) for 7 days also demonstrated reduced ATP and CP levels, increased ADP and decreased AMP (Opie et al., 1979). A recent study utilizing ^{31}P NMR spectroscopy to measure sequential changes in phosphates investigated the role of perfusion duration on ATP and the effect of normal and elevated levels of perfusate long-chain fatty acid on high-energy phosphate metabolism in diabetic heart. Decreased levels of ATP were reported with increased duration of perfusion up to 56 minutes and with increasing palmitate concentrations up to 2.0 mM in the perfusate. Surprisingly, no corresponding decrease was observed for CP. This change was not due to a pH effect in the diabetic heart. Direct chemical analysis of tissue ATP and CP confirmed ^{31}P NMR data (ATP:C = 11.83 ± 1.52 versus D = 7.66 ± 1.43 μmol/gram dry weight) (Pieper et al., 1983).

A variety of mechanisms have been proposed to explain the altered high-energy phosphate levels in diabetic hearts. Pieper et al. (1983), postulated a potential defect in creatine phosphokinase (CPK) in which flux towards CP hydrolysis is inhibited. The observed relationship between reduced ATP and elevated long-chain fatty acyl-CoA may indicate an inhibition of the mitochondrial adenine nucleotide translocase by CoA esters. Functional coupling between the translocase and CPK has been demonstrated at the mitochondrial membrane (Saks et al., 1978) and may be disrupted in diabetes. Specific inhibition of the translocase resulted in a 25% decrease in ATP with no change in CP content

(Pieper et al., 1983). ATP and CP are sensitive indicators of hypoxia and their altered levels may also reflect a disruption in oxygen (O_2) delivery to the heart by erythrocytes (Allison et al., 1976). Diabetic ketoacidosis elicited a 13% decrease in A-V O_2 difference with elevated arterial and venous O_2 contents. Whole blood-O_2 dissociation curves were shifted to the left and correlated with a reduction in 2,3-DPG levels, an effector of hemoglobin-oxygen interaction located in erythrocytes. A decreased 2,3-DPG to hemoglobin molar ratio in the alloxan-diabetic rat would serve to increase blood-oxygen affinity and hence reduce O_2 release. Insulin has been shown to normalize O_2 release. Thus, from this data it was concluded that O_2 delivery to the diabetic myocardium may contribute to reduction in oxidative metabolism in the diabetic heart (Allison et al., 1976).

Increased Heart Work, Anoxia and Ischemia

Normal and diabetic hearts exhibit differing responses to conditions of increased heart work, anoxia and ischemia which further reflect the metabolic aberrations already described. When increased heart work is employed to stimulate glycolytic flux, insulin-deficient hearts demonstrate an impaired ability to increase glucose-uptake relative to the response of normal hearts (Opie et al., 1979). When heart work was increased by increasing atrial filling pressures above 5 cMH_2O diabetic hearts showed depressed peak systolic left ventricular pressure development (LVDP) and cardiac output (Miller, 1979). Increased aortic afterload also elicited a proportional decrease in LVPD and + dP/dt in acutely diabetic hearts but did not influence cardiac output or coronary flow (Ingebretsen et al., 1980). Isoproteronol-stimulated heart work did not elicit different functional responses in diabetic and normal hearts and the authors suggested that catecholamine-stimulated myocardial lipolysis of endogenous substrates may have sustained mechanical function in this condition (Ingebretsen et al., 1980).

Rate and extent of recovery from anoxia were retarded in STZ-diabetic hearts but were improved when insulin and glucose were supplied in the perfusion medium during anoxia. This difference from normal hearts probably pertains to loss of the normal glucose protective effect in transient anoxia. Improved recovery was attributed to stimulated glycolytic flux by insulin leading to anerobic formation of ATP (Opie et al., 1979). Anoxia decreased ventricular pressure to below 20% of basal levels in both control and diabetic hearts. Reoxygenation demonstrated equivalent recoveries in control and diabetics after a 10-minute anoxic exposure, but significantly depressed rates of recovery in diabetic hearts for longer exposures. In addition, increased aortic afterload further reduced the ability of diabetic hearts to recover from 10-minutes of anoxia. Recovery was however aided by high glucose and insulin levels in the perfusate (Ingebretsen et al., 1980). Provision of alternate substrates such as pyruvate, acetate, citrate or β-hydroxybuturate has also been shown to ameliorate diabetic heart recovery from anoxia (Sinclair-Smith, 1979).

In myocardial ischemia effected by coronary artery ligation, chronic diabetes did not influence the severity of ischemia incurred however in acute diabetic ketoacidosis, the ischemic damage was greatly exaggerated (Opie et al., 1979). In mild ischemia, the extent of increased glucose utilization is less for diabetic hearts compared to controls while tissue levels of acyl CoA esters and carnitine accumulate to a greater extent in diabetics suggesting a more severe impairment of oxidative metabolism (Feuvray et al., 1979). At higher workloads and more severe ischemia, ventricular failure occurs more rapidly in diabetic hearts.

Thus, it appears that in hearts not under elevated metabolic demands, energy production from oxidation of lipids compensates for reduced glucose utilization and allows diabetic hearts to maintain near-normal function. However, when demands are increased, the intrinsic inability to generate ATP consequent to increased tissue levels of metabolites from fatty acid oxidation, leads to ventricular failure.

Ketoacidosis

Many of the biochemical characteristics of diabetic ketoacidosis have been described. It is an acute condition which arises in response to insulin deprivation and in the presence of increased levels of glucagon, cortisol, catecholamines and growth hormones leading to aberrant energy metabolism, metabolic acidosis, severe hydration and death. It is characterized also by high blood levels of ketones, acetoacetate and β-hydroxybutarate, which are products of incomplete FFA oxidation and increased ketogenesis in the liver. Figure A-3 illustrates the pathogenesis of ketoacidosis.

Diabetic Cardiomyopathy

Diabetics suffer twice the mortality rate of non-diabetic groups from acute myocardial function and myocardial failure. In the past, coronary atherosclerosis and its complications received endorsement as being responsible for the increased mortality rate. However, most studies on cardiac vessel pathology in diabetes have failed to support this assumption and has led to the concept of a specific diabetic cardiomyopathy (Vihert et al., 1969; Ledet, 1968; Sinclair-Smith, 1979). Pathological, physiological and biochemical data support this concept although the contribution of microvascular pathology to myopathic development is

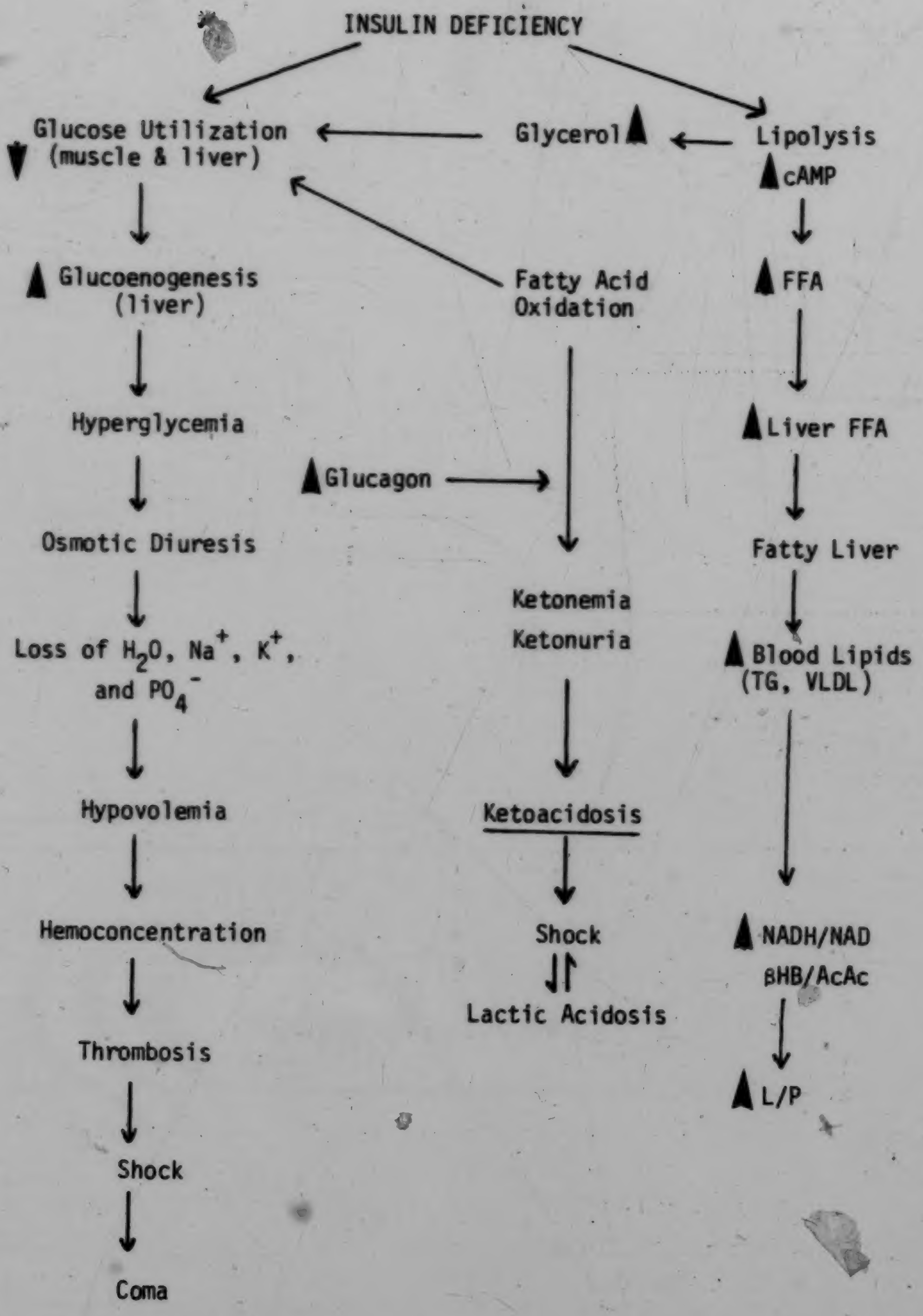
INSULIN DEFICIENCY
▼ Glucose Utilization (muscle & liver)
Glycerol ▲
Lipolysis
▲ cAMP
▲ Glucoenogenesis (liver)
Fatty Acid Oxidation
▲ FFA
Hyperglycemia
▲ Liver FFA
▲ Glucagon
Osmotic Diuresis
Fatty Liver
Ketonemia
Ketonuria
Loss of H_2O, Na^+, K^+, and PO_4^-
▲ Blood Lipids (TG, VLDL)
Hypovolemia
Ketoacidosis
Hemoconcentration
Shock
▲ NADH/NAD
βHB/AcAc
Lactic Acidosis
Thrombosis
▲ L/P
Shock
Coma

Figure A-3. Pathophysiology of ketoacidosis still controversial (Ledet et al., 1979; Strobeck, 1979; Regan et al., 1981). Cardiomyopathies may be of an idiopathic or primary origin or secondary to a known clinical state such as diabetes; although its etiology is not completely understood, diabetic cardiomyopathy is generally considered to be a secondary syndrome (Strobeck et al., 1979).

Research on the etiology and pathogenesis of diabetic cardiomyopathy has been of a diverse nature. Sources have included clinical juvenile and adult diabetics, post-mortem studies and experimental animals with either chemical or spontaneous diabetes. Data has been generated from functional and hemodynamic measurements, histopathological examinations of vascular and non-vascular tissue, isolated working heart models, myocardial fiber contractile mechanics and from biochemical studies.

Vascular Involvement

Early studies described macro- and microvascular alterations in diabetic hearts. Blumenthal et al. (1960) demonstrated that proliferative lesions characterized by accumulation of positive, PAS-staining material and collagen in the endothelium occurred 2.5 times more frequently in diabetics. Atherosclerotic development appeared to occur significantly only in the extramural arteries and branches (Blumenthal et al., 1960; Ledet, 1968; Vihert et al., 1969; Rubler et al., 1972). Several studies have been able to demonstrate a higher incidence of intramural vascular lesions and diffuse microangiopathy in diabetic hearts after varying duration of disease and have therefore suggested that the microangiopathy and impairment of microvascular blood flow may underlie the functional impairment observed in diabetic hearts in the absence of coronary artery disease (Ledet, 1968; Rubler et al., 1972; Hamby et al., 1974; Ledet, 1976; Ledet et al., 1979). Other researchers have been able to record functional impairments in the absence of significant microangiopathy, endothelial thickening or intramural glycoprotein accumulation which has led to the proposal of an extravascular origin of cardiomyopathy (Regan et al., 1977; Crall and Roberts, 1978; Regan and Wiesse, 1978).

Functional Alterations

Hemodynamic studies on diabetic patients have revealed myocardial alterations in function that may result in heart failure as the process develops fully (Regan and Wiesse, 1978). However, no distinctions have yet been made as to the frequency of occurrence in insulin-dependent versus non-insulin dependent patients nor has the importance of disease duration been established. A review of postmortem clinical records of diabetic patients with glomerulosclerosis revealed that 4 out of 27 had demonstrated idiopathic cardiomegaly and congestive heart failure (Rubler et al., 1972). An in situ dog model made mildly diabetic with alloxan provided important insights into cardiomyopathy where end-diastolic volume (EDV) and stroke volume (SV) responses were significantly depressed in the face of increased afterloads as compared to controls. In addition, acute volume expansions elicited much greater end-diastolic pressure (EDP) increments in the diabetic hearts. This altered pressure-volume response was associated with decreased left-ventricular (LV) compliance apparently due to the accumulation of positive PAS-staining glycoprotein material in the interstitium (Regan et al., 1974). Interstitial accumulation of cholesterol and triglyceride (TG) was also recorded. Elevated basal EDV with reduced ejection fractions concomitant with some interstitial glycoprotein accumulation and endocardial fibrosis were reported in some human diabetics (Hamby et al., 1974). Altered systolic time intervals such as a short LV ejection time (LVET), a prolonged pre-ejection period (PEP), increased PEP/LVET ratios and greater isovolumic contraction times may be considered as pre-clinical manifestations of cardiac malfunction in human diabetics (Ahmed et al., 1975; Zoneraich et al., 1977; Rubler et al., 1978; Halder et al., 1978). The altered PEP/LVET ratio indicates a reduced rate of rise of pressure and possible alteration of the myocardial force-velocity relationship in diabetes (Zoneraich et al., 1977). Subnormal stroke work in the face of elevated EDP also indicates LV dysfunction during preload increments (Haider et al., 1978), while a prolonged isovolumic relaxation time indicates further contractile abnormalities (Rubler et al., 1978). Echocardiographic measurements revealed further differences between controls and diabetic in percent of fractional shortening, ejection fraction, end-systolic and end-diastolic diameters and EDV, all of which were indicative of impaired LV function (Zoneraich et al., 1977). Furthermore, elevated interstitial collagen and glycoprotein were documented which suggested that the basis of altered EDP-EDV relationships may be altered LV composition, at least to some extent (Haider et al., 1978). An extensive hemodynamic study on adult-onset diabetes of the familial type without hypertension or obesity compared patients with and without angiographically-proven coronary artery disease (CAD). Left ventricular EDP/EDV ratios were significantly elevated in diabetics in the absence of CAD. The EDV and SV were depressed; the SV attributed to reduced ventricular filling. Afterload increments significantly increased filling pressure with no corresponding increase in SV. Thus, a preclinical cardiomyopathy was evidenced (Regan et al., 1977). Postmortem study on patients having no evidence of CAD revealed again increased interstitial glycoprotein material, collagen deposition in perivascular loci, between myofibers or replacement by fibrosis and enhanced TG and cholesterol accumulation, all suggestive of a diffuse, extravascular abnormality in diabetic cardiomyopathy (Regan et al., 1977). Atrial pacing studies have indicated that the LV ejection abnormality is not due to myocardial ischemia since no increased in lactate production were observed (Regan et al., 1981). Thus, the pathogenesis of diabetic cardiomyopathy has been partially revealed through these studies.

Ultrastructure

Light and electron microscope examination of the myocardium in streptozotocin-diabetic rats (Onishi et al., 1981) and in spontaneously-diabetic mice (Giacomelli and Wiener, 1979) have revealed an extensive pathomorphology in diabetes. Both studies demonstrated diffuse or partial endocardial fibrosis, moderately degenerated myocardial cells, perivascular fibrosis and thickening of the small arteries as well as basement membrane. Cardiac cells initially contained large numbers of lipid droplets which progressed to shrinkage of cells and increased electron dense material in mitochondria enveloped by single membranes which which then gave rise to large residual bodies (Giacomelli and Wiener, 1979). In diabetic rat hearts increased mitochondrial area relative to myofibril area was observed (Onishi et al., 1981). Degeneration of the cardiac myocytes occurred in later stages with partial myofibrilolysis, irregularity of Z-bands, disrupted sarcomere organization and varying degrees of contraction (Giacomelli and Wiener, 1979; Onishi et al., 1981). In addition dilatation of the sarcoplasmic reticulum and focal thickening of the sarcolemma around severely damaged myocytes were observed. These pathomorphological developments were progressive in severity and alterations in cardiac cells preceeded development of significant vascular lesions (Giacomelli and Wiener, 1979). It is interesting to note that cardiomyopathic, non-diabetic hamster hearts revealed similar progressive pathomorphology (Strobeck et al., 1979). The accumulation of large residual bodies and interstitial material has been partly attributed to decreased activity in cardiac lysosomal enzymes (Giacomelli et al., 1980).

Mechanical Performance

Isolated perfused working heart preparations as well as isolated left ventricular papillary muscle models have permitted a more direct, controlled, and quantitative analysis of the effects of drug-induced diabetes on myocardial mechanics. These models are limited insofar as they may not represent the broad spectrum of human diabetic syndromes.

In non-ketotic, hyperglycemia diabetic rats isolated perfused hearts have demonstrated decreased peak left ventricular systolic pressures (PLVSP), and maximum rate of rise of pressure (+dP/dt) with increasing atrial filling pressures (Penparkgul et al., 1980; Vadlamudi and McNeil, 1980 and 1981; Garber and Neely, 1981). In addition, diminished cardiac output, stroke volume, peak aortic flow rate and myocardial oxygen extraction were observed with increasing workloads in diabetic hearts relative to controls (Penparkgul et al., 1980). Basal levels of LV pressure development and max + dP/dt have been reported to be similar between the two groups (Vadlamudi and McNeil, 1980). Rates of cardiac relaxation (negative dP/dt) were decreased at all workloads (Penparkgul et al., 1980; Vadlamudi and McNeil, 1981). The EDP/normalized EDV curve was shifted to the right in diabetics. Provision of increased glucose in the perfusate or glucose plus insulin did not rectify the abnormalities in LV pressure and dP/dt. In addition, insulin alone showed no normalizing effect on these parameters (Penparkgul et al., 1980). Cardiac response to isoproterenol regarding LVSP and +dP/dt were not different in diabetics (Vadlamudi and McNeil, 1980). However, diabetic hearts did demonstrate an increased sensitivity to the negative ionotropic effect of carbachol where LVSP decreased to a greater extent in diabetics (Vadlamudi and McNeil, 1981). In general, diabetic hearts demonstrate an intrinsic decreased ability to maintain function at higher workloads. Cardiomyopathic, non-diabetic hamster hearts were reported to demonstrate similar dysfunction (Strobeck et al., 1979).

Work with isolated papillary muscle supports the data on whole hearts and further characterizes the cardiomyopathic response (Fein et al., 1980). Chronic streptozotocin-induced diabetes resulted in significant declines in rate of tension development and rates of relaxation at all workloads (Fein et al., 1980; Heyliger et al., 1982; Strobeck et al., 1979). After 5, 10 and 30 weeks of diabetes, no differences between control and diabetic muscle passive and active length-tension curves were observed. However, the relationship between muscle length and peak rate of tension decline was observed to be depressed in diabetics over a large part of the muscle lengths studied (Fein et al., 1980). Isotonic studies revealed prolonged time to peak shortening, time to peak relaxation velocity and lower peak shortening and lengthening velocities. The force-velocity relationship of diabetic hearts was depressed. Low bath concentrations of Ca^{2+} (0.6 mM) showed no differences between control and diabetic muscle, however at higher bath Ca^{2+} (2.4 mM) the differences in shortening and relaxation velocities became apparent. Measurements of series elasticity revealed no differences between the two groups (Fein et al., 1980). Increasing bath glucose concentration from 0.10

to 1.0 grams/100 ml produced no relative differences in the responses of control and diabetic hearts as regards developed tension and time to one-half relaxation. Varied stimulus frequency also did not alter the previously noted responses of control and diabetic hearts. Norepinephrine exhibited a major effect on relaxation time in diabetics where rates were depressed for diabetics at all norepinephrine concentrations, but had no effect on developed tension. The lack of norepinephrine effect on tension decline may relate to the decreased density of β-receptors found on diabetic papillary muscle (Heyliger et al., 1982). In summary, diabetic isolated papillary muscle demonstrated delayed onset of relaxation, a slowed rate of relaxation, delays in attaining peak isometric and isotonic relaxation rates, slowed rates of tension development and a depressed force-velocity relationship. Peak developed tension and peak shortening velocity was not altered in diabetes and it was put forward that decreased corresponding rates were incurred in an attempt to compensate for declining contractility in the diabetic heart (Fein et al., 1980).

Biochemical Contractile Alterations

The observed functional and mechanical alterations observed in the diabetic heart may be the result of abnormalities in biochemical indices of contractile behaviour. Significant reductions in the cardiac myofibril ATPase response have been reported with STZ- and alloxan-diabetes over a range of 2 days to 8 weeks (Garber and Neely, 1981; Pierce and Dhalla, 1981; Malhotra et al., 1981; Dillman, 1980). Basal, Mg^{2+}-stimulated myofibril ATPase was depressed in diabetic hearts at 8 weeks (0.16 ± 0.02 versus 0.22 ± 0.01 μ mol $Pi \cdot mg^{-1} \cdot 5\ min^{-1}$) (Pierce and Dhalla, 1981).

Ca^{2+}-activated myofibril ATPase activity was impaired in diabetics relative to controls over a range of free Ca^{2+}-concentrations of 0.10 to 10 µM Ca^{2+} (pCa 7 to 5). Normalization of the two curves relative to their respective maximal activities revealed a similar Ca^{2+}-dependence on the diabetic and control myofibrils (Pierce and Dhalla, 1981). Ca^{2+}-activated actomyosin ATPase was significantly depressed at 1 week post-injection and reached a 60% reduction at 2 weeks (Malhotra et al., 1981) while a 35% decrease was reported for 4 weeks (Dillman, 1980). Similar results were noted for Ca^{2+}-myosin ATPase activity where significant depressions occurred at 2 (Malhotra et al., 1981; Garber and Neely, 1981) and at 4 weeks (Dillman, 1980). Actin-activation of Mg^{2+}-stimulated myosin ATPase was also reduced from the 11-fold activation of controls to an 8-fold activation in diabetics (Malhotra et al., 1981). Unlike other conditions, the K^{+}-EDTA myosin ATPase was enhanced in diabetics.

Responses to varying KCl concentrations, which disrupts protein structure, were also different in diabetic hearts. Basal ATPase of diabetics demonstrated a progressive decline in activity from 100 to 300 mM KCl, while after an initial decrease at 100 mM KCl, the control values plateaued. At maximal Ca^{2+}-activation both control and diabetic myofibrils showed similar patterns of inhibition with increasing KCl although the extent of decrease was significantly greater at 100 and 200 mM KCl in the diabetics. The authors suggested that these alterations may represent fundamental differences in protein structure in diabetes (Pierce and Dhalla, 1981).

Involvement of thiol groups in the reduced ATPase of diabetic myofibrils has also been implicated. Iodoacetamide, which influences the SH_1 and SH_2 groups of myosin, elevated the Ca^{2+}-ATPase activity of myosin by 170% in diabetics as compared to only 41% in controls

(Malhotra et al., 1979). In addition, ethylene glycol, which detects informational changes at or near the myosin active site, elicited a differential response in diabetics. Basal ATPase exhibited a greater sensitivity to ethylene glycol with diabetes whereas Ca^{2+}-stimulated ATPase activities were similar (Pierce and Dhalla, 1981). Diabetes therefore may modify thiol groups associated with the myosin active site.

A shift in the myosin isoenzyme distribution with diabetes has been documented and offers one explanation for the altered ATPase responses. Dillman (1980) demonstrated that the distribution pattern shifted from a V_1-predominant pattern (72% to 15%) with its greater ASPase activity to a V_3-domination (15% to 68%) and its 10-fold lower activity. A similar redistribution was noted by Malhotra et al., (1981). It was suggested that this change alone could account for the altered Ca^{2+}-ATPase activity in diabetes (Dillman, 1980). Insulin adminstration normalized both the isoenzyme pattern and ATPase response (Dillman, 1980).

Reduced levels of thyroid hormone (T_3 and T_4) concomitant with diabetes have also been observed and may influence ATPase activities (Fein et al., 1980; Dillman, 1980; Malhotra et al., 1981; Dillman, 1981). Throidectomized rats displayed greater reductions in the Ca^{2+}-ATPase activities of myosin and actomyosin than diabetics alone, while thyroid replacement in diabetics did not alleviate the depressed ATPase response (Malhotra et al., 1981). However, Dillman (1980) demonstrated that pharmacological but not physiological doses of thyroid T_3 hormone successfully normalized the ATPase and myosin isoenzyme patterns in diabetic rats. This response indicated a diminished thyroid hormone responsiveness in diabetic rat or could reflect diabetes-related cellular disturbances which are sensitive to high doses of thyroid hormone (Dillman, 1981). The involvement of thyroid hormone in diabetes is still unclear.

That altered myofibril ATPase responses are indicative of a cardiomyopathic condition is supported by studies on non-diabetic cardiomyopathic hamsters where significantly depressed myosin and actomyosin ATPase activities were recorded during various stages of the disease leading to ventricular failure (Strobeck et al., 1979; Pang and Weghicki, 1980).

Diabetes also disrupts other cellular components of the excitation-contraction mechanism. Depressed Ca^{2+}-uptake and Mg^{2+}-ATPase and Ca^{2+}-Mg^{2+} ATPase activities in the sarcoplasmic reticulum have been observed (Penparkgul et al., 1981). This may potentially explain the defective relaxation mechanics noted in isolated whole hearts and papillary muscles (Penparkgul et al., 1980; Fein et al., 1980). In addition, the Na^{2+}-K^{+} ATPase activity of sarcolemma and its insulin-induced increase were depressed (46.3%) in diabetic ventricular papillary muscle. Resting membrane potential and hyperpolarization by insulin were less negative and reduced in the diabetic preparation (Imanaga et al., 1981). Similarly, Onji and Liu (1980) reported a decrease in the K^{+} affinity of the Na^{+}-K^{+} ATPase enzyme system. Thus, comprehensive disturbances in contractile function have been documented in experimental diabetes.

APPENDIX B

LABORATORY PROCEDURES

APPENDIX B-1

Preparation and Injection of Streptozotocin (STZ)

1. Prepare citrate buffer (pH 4.5) from stock solutions:

citric acid (0.10 M)	24.5 mls
sodium citrate (0.10 M)	25.5 mls

Make up to 100 mls with distilled H_2O.
Keep covered in refridgerator.

2. Prepare STZ (< 24 hours before use):

For 60 $mg \cdot kg^{-1}$ dosage:

a) weigh out 60 mg STZ per animal into small tubes.
b) cover with Parafilm.
c) keep on ice during weighing; store @ -80°C until use.

Immediately prior to injection:

3. Determine animal body weights (BW).

4. Calculate volume of STZ-buffer injection for 60 $mg \cdot kg^{-1}$ dosage:

Take animal BW x 0.001 = volume of injection (mls).
(i.e.: 200 g x 0.001 = 0.2 mls of STZ-buffer)

5. STZ-buffer solution:

Pipette 1 ml citrate buffer (pH 4.5) into tube with 60 mg STZ.
Vortex well.

Therefore:

0.20 mls x 60 mg = 12 mg injected into a 200 g animal

Final dose: $\frac{12 \text{ mg}}{200 \text{ g}} = \frac{X \text{ mg}}{1000 \text{ g}} = 60 \text{ mg} \cdot \text{kg}^{-1}$.

6. While preparing STZ-buffer solution anesthetize animal in ether chamber.

7. Take up required volume plus extra into a surgical 28½ guage needle. Remove all air bubbles. Bend bevelled tip of needle to ~90°. Remove excess volume.

8. Remove unconcious animal from chamber. Lay on back, expose penile dorsal view visible just below skin surface.
With bevelled edge up, insert needle into vein almost horizontally for most of needle length. Inject STZ slowly. Wipe off excess. If ballooning or excess bleeding occurs discontinue injection immediately.

9. Animal should regain consciousness soon after injection.

*NOTE: Due to toxicity of STZ, wear surgical gloves when handling.

APPENDIX B-2

Isolation of Myofibril Proteins from Cardiac Muscle
(Perry and Corsi, 1958, with modifications)

Reagents and Chemicals

1. Buffer I: 39 mM Na-Borate; 25 mM KCL; 5 mM EDTA (ethylenediamine-tetracetic acid); pH 7.1.

   EDTA chelates divalent cations Ca^{2+} and Mg^{2+} thereby inducing relaxation.

2. Buffer II: 39 mM Na-Borate; 50 mM Tris; pH 7.1.

   Washes out EDTA and cations.

3. Wash Solution: 50 mM Tris; 10 mM NaN_3 (sodium azide; inhibits mitochondrial ATPase): 100 mM KCL; 0.5% Triton X-100 detergent (removes membrane-bound SR and sarcolemmal ATPases; Solaro et al., 1971).

4. Suspension Medium: 150 mM KCL; 50 mM Tris; pH 7.4.

All solutions are pH'ed at 4°C.

Procedure

1. Weigh tissue.

2. Homogenize tissue in 20 volumes of cold Buffer I for 20 seconds at a setting of 7 with a Polytron Pt-10 tissue homogenizer.

3. Centrifuge the homogenate at 1000 xg for 10 minutes.

4. Decant supernatant and discard. Resuspend the pellet in 20 volumes of cold Buffer I, centrifuge at 1000 xg for 10 minutes and discard supernatant.

5. Resuspend pellet in 20 volumes of cold Buffer II, centrifuge at 2200 rpm for 10 minutes and discard supernatant.

6. Repeat step 5.

7. Resuspend pellet in 20 volumes of cold Wash solution, centrifuge at 1000 xg for 10 minutes and discard supernatant.

8. Repeat step 7.

9. Resuspend pellet in 20 volumes of cold Suspension Medium, centrifuge at 1000 xg for 10 minutes and discard supernatant.

10. Resuspend pellet in 5 ml Suspension Medium.

11. Take 0.1 ml for determination of protein concentration (Lowry et al., 1951).

APPENDIX B-3

Protein Determination (Lowry et al., 1951)

Reagents and Chemicals

1. 0.5% Cupric Sulphate ($CuSO_4.5H_20$)
2. 1.0% Sodium Potassium Tartrate ($NaKC_4H_4O_6$)
3. 2.0% Sodium Carbonate (Na_2CO_3) pH to 12.5 with 10 N NaOH at room temperature.
4. Lowrey C Solution: 61.7% $NaCO_3$

   35.7% Deionized Water

   1.3% $CuSO_4$

   1.3% $NaKC_4H_4O_6$
5. Folin Reagent: 1 to 1 (v/v) dionized water
6. 0.3 N KOH
7. Suspension Medium (see Myofibril Isolation)

Standard Curve (Protein Stock Solution: 5 mg/ml of bovine serum albumin in distilled water)

Stock (ml)	Suspension Medium (ml)	Concentration (mg/ml)	Final Concentration (ug/ml)
0.0	0.5	0.0	0
0.1	0.4	1.0	6.0
0.2	0.3	2.0	12.0
0.3	0.2	3.0	19.0
0.4	0.1	4.0	25.0
0.5	0.0	5.0	30.0

Procedure

a) Solubilizing Protein

- i) Take 0.1 ml of homogenate or standard solution.
- ii) Add 0.2 ml of 0.3 N KOH.
- iii) Incubate in water bath at 37°C for 30 minutes.

b) Reaction Mixture

- i) Take 0.1 ml (twice) of solubolized protein from above.
- ii) Add 5.0 ml of freshly prepared Lowry C Solution to duplicate tubes.
- iii) Add 0.3 ml of folin reagent of each tube while vortexing. Insure equal duration of mixing for all tubes.
- iv) Allow reaction mixture to stand for at least 45 minutes.

c) Spectrophotometric Analysis

- i) Set spectrophotometer wavelength at 750 nm (Pye-Unicam SP8-100).
- ii) Use protein blank from the standard curve as the reference.
- iii) Vortex each tube before the analysis of each standard and sample tube. Record optical density.

APPENDIX B-4

Incubation for Myofibril ATPase Activity
(Belcastro et al., 1980)

Reagents and Chemicals

1. Reaction medium (pH 7.0; 30°C) 100 mM KCl
   4 mM Tris-base
   2.0 mM $MgCl_2$

2. Calcium-EGTA:

   Maximal activation (10 μM free Ca^{2+}) (pH 7.0; 30°C) 1 mM EGTA
   $CaCl_2$

   Maximal activation (10 μM free Ca^{2+}) (pH 6.5; 30°C) 1 mM EGTA
   $CaCl_2$

   Basal, Mg^{2+}-stimulated (0 free Ca^{2+})
   (pH 7.0; 30°C)
   (pH 6.5; 30°C) 5 mM EGTA

3. 23% Trichloroacetic Acid (12% TCA)

4. 50 mM MgATP (pH 7.0; 30°C) 0.1632 g ATP
   0.0508 g $MgCl_2$
   5 ml deionized H_2O

5. Suspension Medium (see B-2)

6. For Inorganic Phosphate conditions only:

   0.10 mM KH_2PO_4 (pH 7.0; 30°C)
   1.0 mM KH_2PO_4 (pH 7.0; 30°C)

7. For Acidosis condition only:

   Adjust solutions #1, 4, and 6 to pH 6.5 at 30°C with dilute HCl.

Procedure

A. Reaction Medium (everything kept on ICE)

   1. Pipette 0.50 ml of reaction medium into both A (specific) and X (non-specific activity) tubes.

   2. Pipette 0.05 ml Ca^{2+}-EGTA solution into each tube.

   3. Taking into account the later addition of 0.10 mM MgATP solution

make each tube up to 1.0 ml with addition of 0.1 ml Suspension Medium.

(NOTE: For Pi procedure substitute 0.1 ml KH_2PO_4 solution for suspension medium)

4. Add 1.0 ml 12% TCA to very X tube.

B. Incubation:

1. After adjusting protein to 2 mg·ml^{-1}, add 0.25 ml protein to each tube at 15 second intervals.
2. Vortex well and incubate tubes at 30°C for exactly 5 minutes.
3. At 15 second intervals, vortex tube, ADD 0.1 ml MgATP solution to each tube and incubate again for exactly 5 minutes at 30°C. Vortex throughout 5 minutes.
4. At 15 second intervals remove tubes from bath and place immediately on ICE.

   For A tubes: Stop reaction with addition of 1 ml cold 12% TCA, vortex and place in ice.

   For X tubes: Vortex and place on ICE.
5. Allow tubes to stand at least 10 minutes.
6. Centrifuge 10 minutes at 1000 xg.
7. Decant supernatant and save for phosphate determination.

APPENDIX B-5

Phosphate Determination

Reagents and Chemicals

1. 10 N Sulphuric Acid: 28 ml H_2SO_4 (98%) made up to 100 ml with deionizer water.

2. Ammonium Molybdate - Ferrous Sulphate Solution: take 0.5 g ammonium molybdate and completely dissolve in 5.0 ml 10 N H_2SO_4. Bring up to 30.0 ml with deionizer water. Add 2.5 g Ferrous Sulphate and dissolve. Bring up to 50 ml with deionized water.

Standard Curve (Phosphate Stock Solution: 1.0 mM KH_2PO_4)

Stock (ml)	Water (ml)	Final Concentration (μ moles)
1.00	0.00	1.00
0.50	0.50	0.50
0.10	0.90	0.10
0.05	0.95	0.05
0.01	0.99	0.01
0.00	1.00	0.00

Procedure

1. To blank and standard tubes add 1.5 ml 12% TCA.

2. To sample tubes (A and X) add 1.0 ml 12% TCA.

3. Remove 0.050 ml (50 ul) from standard tubes and replace with 0.050 ml of KH_2PO_4 (phosphate standard).

4. To sample tubes add 0.5 ml supernatant from centrifugation.

5. Add 1 ml of ammonium molybdate - ferrous sulphate solution to all tubes vortex and let stand for exactly 10 minutes.

Spectrophotometric Analysis

1. Set spectrophotometer to wavelength 700 nm (Pye-Unicam SP8-100).

2. Use deionized water as the reference.

3. Record optical density.

4. Calculate ATPase activity according to the following equation:

$$\frac{\dfrac{4 \times .05\ [\text{activated sample OD} - \text{nonspecific activity}]/\text{standard OD}}{0.5\ \text{mg protein}}}{5\ \text{min.}}$$

APPENDIX B-6

RELIABILITY DETERMINATIONS ON BIOCHEMICAL PROCEDURES

Procedure	# Trials*	Coefficient of Variation
1. Pipetting		
1.00 ml	20	2.76%
0.50 ml	20	1.06%
0.20 ml	20	0.343%
0.10 ml	20	0.599%
0.050 ml	20	3.57%
2. Lowry Protein Assay from myofibril isolation	20	2.42%
3. Phosphate Standard Curve (Taussky and Shorr, 1953)		
0.50 µmol Pi·ml^{-1}	10	1.19%
0.10 µmol Pi·ml^{-1}	10	1.68%
0.05 µmol Pi·ml^{-1}	10	1.62%
0.01 µmol Pi·ml^{-1}	10	3.17%
4. Myofibril ATPase at 10 µM Ca^{2+}	20	2.77%
+ Phosphate Determination	20	3.20%

*: Assays done in duplicate

APPENDIX C

RAW DATA

APPENDIX C

TABLE C-1

CUMULATIVE SWIM TIMES (HOURS) OVER EIGHT WEEK TRAINING PERIOD

Training Non-Diabetic		Training Diabetic	
Animal	Total Swim Time	Animal	Total Swim Time
43	30.75 hrs.	122	30.75 hrs.
63	30.75	130	30.75
44	30.75	117	28.30
70	30.75	124	30.60
38	28.40	136	30.73
85	28.80	126	30.43
90	29.40		
$\overline{X}$	29.94 hrs.	$\overline{X}$	30.26 hrs.

APPENDIX C

TABLE C-2

WEEKLY BODY WEIGHTS (GRAMS)

	Animal	Initial	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Prior to Sacrifice Week 9
SND	51	262	344	367	391	413	437	435	473	484	451
	54	250	334	369	386	407	434	453	473	490	483
	57	303	359	385	406	428	453	443	481	501	474
	65	302	373	394	406	448	469	475	489	495	499
	46	266	356	382	353	373	401	403	436	430	434
	92	304	358	372	401	410	432	443	468	466	459
	80	293	348	379	401	425	453	446	478	484	481
SD	103	236	252	265	275	252	223	235	247	246	255
	107	240	198	240	241	235	218	212	221	221	219
	109	249	223	274	287	272	264	279	282	283	291
	138	231	196	256	268	260	239	254	248	245	248
	112	227	277	314	326	330	305	295	310	323	323
	143	227	307	340	372	341	367	356	381	410	412

APPENDIX C-2 (Continued)

	Animal	Initial	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Prior Sacrifice Week 9
TC	43	253	328	347	349	370	385	400	404	415	416
	63	290	368	360	380	380	393	364	389	408	416
	94	305	349	360	378	381	396	385	410	415	422
	70	286	348	359	370	387	401	405	416	428	426
	38	302	375	391	403	420	431	447	456	455	456
	85	306	373	379	384	402	427	410	424	370	380
	90	281	347	359	371	375	394	410	406	416	419
TD	122	237	284	318	339	315	333	310	299	295	315
	130	216	269	303	311	324	334	326	342	338	370
	117	237	298	318	329	334	357	306	323	275	267
	124	233	240	258	260	237	240	226	223	215	229
	136	221	286	315	315	319	291	272	291	281	305
	126	229	293	310	327	309	303	292	304	295	324

APPENDIX C

TABLE C-3

RAW HEART WEIGHT-BODY WEIGHT DATA

Group	Animal	mg Vent. Weight	grams Body Weight	V.W.:B.W.
SC	51	1236 mg	451 g	2.74
	54	1145	483	2.37
	57	1167	474	2.46
	65	1076	499	2.15
	46	1244	434	2.86
	92	1015	459	2.21
	80	1135	481	2.53
SD	103	850	255	3.33
	107	747	219	3.41
	109	878	291	3.01
	138	702	248	2.83
	112	952	323	2.94
	143	988	412	2.39
TC	43	1077	416	2.58
	63	1161	416	2.79
	44	1147	422	2.71
	70	1143	426	2.68
	38	1126	456	2.46
	85	1186	380	3.12
	90	1042	419	2.48
TD	122	826	315	2.62
	130	1080	370	2.91
	117	765	267	2.86
	124	687	229	3.0
	136	963	305	3.15
	126	903	324	2.78

APPENDIX C

TABLE C-4

RAW PLASMA GLUCOSE DATA (NON-FASTING)

Group	Animal	Glucose mg/dl		
		Week 1	Week 4	Week 8
SC	42	138	123	124
SC	61	120	122	117
SC	54	136	139	110
SC	46	125	121	121
SC	92	124	116	117
SC	80	135	131	142
SD	103	684	1002	680
SD	107	792	717	549
SD	109	882	762	765
SD	138	1172	852	648
SD	112	702	768	771
SD	143	474	573	672
TD	122	711	615	570
TD	130	534	774	870
TD	117	478	423	410
TD	124	897	816	702
TD	136	741	558	825
TD	126	630	598	705

APPENDIX C

TABLE C-5

PLASMA URINE VALUES

Group	Animal	Glucose mg/dl	
		Week 4	Week 8
SC	54	-	200.5
	46	-	61.5
	92	-	420
	80	-	155
	61	-	166
SD	103	-	2,940
	107	5,943	-
	109	7,770	10,720
	138	8,043	7,940
	112	7,456	9,390
	143	13,209	10,843
TD	122	7,518	8,200
	130	4,021	3,876
	117	1,875	6,892
	124	6,919	6,180
	136	6,006	6,936
	126	5,082	6,482

APPENDIX C

TABLE C-6

MYOFIBRIL PROTEIN YIELDS

	Control		Diabetic	
Animal	Sedentary	Trained	Sedentary	Trained
1	35.21	38.29	33.67	36.12
2	34.35	34.74	35.80	31.29
3	34.79	37.33	33.39	35.80
4	37.98	32.75	35.14	42.75
5	85.43	35.91	30.38	40.35
6	37.10	33.90	32.80	32.70
7	31.42	32.68	-	-
$\overline{X}$	35.12	35.08	33.53	36.50
± S.E.	±0.89	±0.83	±0.78	±1.78

Units: mg myofibril protein/gram wet tissue weight

APPENDIX C

TABLE C-7

Ca^{2+}-ACTIVATED CARDIAC MYOFIBRIL ATPASE ACTIVITIES*

IN VITRO CONDITION		GROUP			
pH	phosphate (KH_2PO_4)	SC	TC	SD	TD
7.0	0	0.084 ± 0.002	0.097 ± 0.005	0.042 ± 0.005	0.048 ± 0.005
7.0	0.10 mM	0.112 ± 0.005	0.112 ± 0.005	0.055 ± 0.005	0.062 ± 0.004
7.0	1.0 mM	0.084 ± 0.005	0.108 ± 0.008	0.054 ± 0.003	0.048 ± 0.005
6.5	0	0.032 ± 0.005	0.032 ± 0.005	0.017 ± 0.003	0.021 ± 0.003
6.5	0.10 mM	0.048 ± 0.005	0.069 ± 0.011	0.024 ± 0.004	0.029 ± 0.003
6.5	1.0 mM	0.031 ± 0.006	0.047 ± 0.004	0.020 ± 0.002	0.025 ± 0.007

*Activity expressed as μmol Pi·mg^{-1}·min^{-1} ($\bar{X}$ ± S.E.)

APPENDIX C

TABLE C-8

BASAL (Mg^{2+}-STIMULATED) CARDIAC MYOFIBRIL ATPASE ACTIVITIES*

IN VITRO CONDITION		GROUP			
pH	phosphate (KH_2PO_4)	SC	TC	SD	TD
7.0	0	0.010 ± 0.001	0.018 ± 0.002	0.011 ± 0.001	0.008 ± 0.001
7.0	0.10 mM	0.035 ± 0.002	0.019 ± 0.003	0.014 ± 0.003	0.013 ± 0.002
7.0	1.0 mM	0.018 ± 0.003	0.008 ± 0.001	0.010 ± 0.002	0.007 ± 0.001
6.5	0	0.013 ± 0.003	0.018 ± 0.004	0.009 ± 0.001	0.011 ± 0.002
6.5	0.10 mM	0.020 ± 0.003	0.025 ± 0.006	0.019 ± 0.004	0.016 ± 0.003
6.5	1.0 mM	0.029 ± 0.006	0.014 ± 0.003	0.009 ± 0.001	0.012 ± 0.001

*Activity expressed as μmol $Pi \cdot mg^{-1} \cdot min^{-1}$ ($\overline{X} \pm$ S.E.)

APPENDIX D

STATISTICAL PROCEDURES

APPENDIX D

TABLE D-1

ONE-WAY ANOVA ON PLASMA GLUCOSE LEVELS WITH REPEATED MEASURES ON TIME (1, 4, 8 WEEKS) FOR CONTROL VERSUS SEDENTARY DIABETICS

Source of Variation	Sum of Squares	Mean of Squares	Degrees of Freedom	F Ratio	Tail Probability
Groups	3486933.7	3486933.7	1	194.64	0.0000
Error	179145.2	17914.2	10		
Time	22824.6	11412.3	2	0.98	0.3892
Time-Group	18114.8	9057.4	2	0.79	0.4695
Error	230614.4	11530.7	20		

APPENDIX D

TABLE D-2

ONE-WAY ANOVA ON PLASMA GLUCOSE LEVELS WITH REPEATED MEASURES ON TIME (1, 4, 8 WEEKS) FOR SEDENTARY VERSUS TRAINED DIABETICS

Source of Variation	Sum of Squares	Mean of Squares	Degrees of Freedom	F Ratio	Tail Probability
Groups	71824.0	71824.0	1	1.74	0.2171
Error	413808.5	41380.8	10		
Time	11741.7	5870.8	2	0.33	0.7239
Time-Groups	36787.2	18393.5	2	1.03	0.3757
Error	357641.1	17882.1	20		

APPENDIX D

TABLE D-3

TWO-WAY ANOVA ON VENTRICULAR WEIGHTS IN CONTROL AND DIABETIC, SEDENTARY AND SWIM-TRAINED ANIMALS

Source of Variation	Sum of Squares	Mean of Squares	Degrees of Freedom	F Ratio	Tail Probability
Training	4.3	4.3	1	0.00	0.9828
Diabetes	525258.0	525258.0	1	57.74	0.0000
T-D	2432.8	2432.8	1	0.27	
Error	218320.0	9096.6	24		

APPENDIX D

TABLE D-4

TUKEY POST-HOC COMPARISONS OF VENTRICULAR WEIGHT MEANS

	SC	TC	SD	TD
SC	-	-	292.7 ($p < 0.001$)	-
TC		-	-	255.3 ($p < 0.001$)
SD			-	-
TD				-

Critical Difference

$p < 0.05$: 140.56g

$p < 0.001$: 176.96g

SC = sedentary control. TC = trained control.

SD = sedentary diabetic. TD = trained diabetic.

APPENDIX D

TABLE D-5

TWO-WAY ANOVA ON BODY WEIGHTS OF CONTROL AND DIABETIC, SEDENTARY AND SWIM-TRAINED ANIMALS

Source of Variation	Sum of Squares	Mean of Squares	Degrees of Freedom	F Ratio	Tail Probability
Training	2661.75	2661.75	1	1.53	0.2279
Diabetes	152293.75	152293.75	1	87.63	0.0000
T-D	6270.03	6270.03	1	3.61	0.0696
Error	41711.71	1737.98	24		

APPENDIX D

TABLE D-6

TUKEY POST-HOC COMPARISONS OF BODY WEIGHT MEANS

	SC	TC	SD	TD
SC	-	49. $(p > 0.05)$	177.4 $(p < 0.001)$	-
TC		-	-	117.6 $(p < 0.001)$
SD			-	10.4 $(p > 0.05)$
TD				-

Critical Difference

$p < 0.05$: 61.45g

$p < 0.001$: 77.38g

SC = sedentary control. TC = trained control.

SD = sedentary diabetic. TD = trained control.

APPENDIX D

TABLE D-7

TWO-WAY ANOVA ON HEART WEIGHT: BODY WEIGHT RATIOS OF CONTROL AND DIABETIC, SEDENTARY AND SWIM-TRAINED ANIMALS

Source of Variation	Sum of Squares	Mean of Squares	Degrees of Freedom	F Ratio	Tail Probability
Training	0.02343	0.02343	1	0.36	0.5538
Diabetes	0.88218	0.88218	1	13.58	0.0012
T-D	0.17129	0.17129	1	2.64	0.1175
Error	1.55937	0.06497	24		

APPENDIX D

TABLE D-8

TUKEY POST-HOC COMPARISONS OF HW:BW RATIO MEANS

	SC	TC	SD	TD
SC	-	0.21 ($p > 0.05$)	0.41 ($p < 0.05$)	-
TC		-	-	0.20 ($p < 0.05$)
SD			-	0.10 ($p > 0.05$)
TD				-

Critical Difference

$p < 0.05$: 0.37

$p < 0.001$: 0.47

SC = sedentary control. TC = trained control.

SD = sedentary diabetic. TD = trained diabetic.

APPENDIX D

TABLE D-9

FOUR-WAY ANOVA ON Ca^{2+}-ACTIVATED CARDIAC MYOFIBRIL ATPASE ACTIVITY WITH REPEATED MEASURES ON TWO FACTORS (pH AND Pi)

Source of Variation	Sum of Squares	Mean of Squares	Degrees of Freedom	F Ratio	Tail Probability
Diabetes (D)	0.04869	0.04869	1	89.62	0.0000
Training (T)	0.00262	0.00262	1	4.83	0.0376
D-T	0.00081	0.00081	1	1.50	0.2326
Error	0.01304	0.00054	24		
pH	0.07663	0.07663	1	673.82	0.0000
pH-D	0.00809	0.00809	1	71.16	0.0000
pH-T	0.00002	0.00002	1	0.20	0.6578
pH-D-T	0.00001	0.00001	1	0.07	0.7967
Error	0.00273	0.00011	24		
Pi	0.00902	0.00451	2	55.89	0.0000
Pi-D	0.00149	0.00075	2	9.26	0.0004
Pi-H	0.00011	0.00006	2	0.71	0.4989
Pi-D-H	0.00071	0.00036	2	4.42	0.0172
Error	0.00387	0.00008	48		

TABLE D-9 (Continued)

Source of Variation	Sum of Squares	Mean of Squares	Degrees of Freedom	F Ratio	Tail Probability
pH-Pi	0.00000	0.00000	2	0.01	0.9924
pH-Pi-D	0.00020	0.00010	2	0.85	0.4343
pH-Pi-T	0.00049	0.00024	2	2.10	0.1330
pH-Pi-D-T	0.00089	0.00044	2	3.84	0.0284
Error	0.00554	0.00012	48		

Pi - Inorganic Phosphate

APPENDIX D

TABLE D-10

TUKEY POST-HOC COMPARISONS OF Ca^{2+}-ACTIVATED MYOFIBRIL ATPASE ACTIVITIES
(pH 7.0: Within and Between Group Differences)

	A_1	A_2	A_3	B_1	B_2	B_3	C_1	C_2	C_3	D_1	D_2	D_3
A_1	-	0.028 **		0.013			0.042 **					
A_2		-	0.028 **		0			0.057 **				
A_3			-			0.024 *			0.030 **			
B_1				-	0.015 *	0.011				0.049 **		
B_2					-	0.004					0.050 **	
B_3						-						0.060 **
C_1							-	0.014		0.006		
C_2								-			0.007	
C_3									-			0.006
D_1										-	0.014	0
D_2											-	0.014
D_3												-

TABLE D-10 (Continued)

Critical Difference

$p < 0.05$: 0.015 $\mu mol\ Pi \cdot mg^{-1} \cdot min^{-1}$ (*)
$p < 0.001$: 0.018 $\mu mol\ Pi \cdot mg^{-1} \cdot min^{-1}$ (**)

A = Sedentary Control (SC)
B = Trained Control (TC)
C = Sedentary Diabetic (SD)
D = Trained Diabetic (TD)

1 = 0 mM Pi
2 = 0.10 mM Pi
3 = 1.0 mM Pi

APPENDIX D

TABLE D-11

TUKEY POST-HOC COMPARISONS ON Ca^{2+}-ACTIVATED MYOFIBRIL ATPASE ACTIVITIES
(pH 6.5: Within and Between Group Differences)

	A_1	A_2	A_3	B_1	B_2	B_3	C_1	C_2	C_3	D_1	D_2	D_3
A_1	-	0.016*	0.001	0			0.015*					
A_2		-	0.017*		0.021**			0.024**				
A_3			-			0.016*			0.011			
B_1				-	0.037**	0.016*				0.011		
B_2					-	0.021**					0.040**	
B_3						-						0.022**
C_1							-	0.007	0.003	0.004		
C_2								-	0.004		0.005	
C_3									-			0.005
D_1										-	0.008	0.004
D_2											-	0.004
D_3												-

TABLE D-11 (Continued)

Critical Difference

$p < 0.05$: 0.015 μmol Pi·mg^{-1}·min^{-1} (*)
$p < 0.001$: 0.018 μmol Pi·mg^{-1}·min^{-1} (**)

A = SC
B = TC
C = SD
D = TD

1 = 0 mM Pi
2 = 0.10 mM Pi
3 = 1.0 mM Pi

APPENDIX D

TABLE D-12

FOUR-WAY ANOVA ON BASAL, Mg^{2+}-STIMULATED CARDIAC MYOFIBRIL ATPASE ACTIVITY WITH REPEATED MEASURES ON TWO FACTORS (pH AND Pi)

Source of Variation	Sum of Squares	Mean of Squares	Degrees of Freedom	F Ratio	Tail Probability
Diabetes (D)	0.00226	0.00026	1	21.89	0.0001
Training (T)	0.00021	0.00021	1	2.08	0.1619
D-T	0.00008	0.00008	1	0.80	0.3790
Error	0.00248	0.00010	24		
pH	0.00017	0.00017	1	3.41	0.0771
pH-D	0.00001	0.00001	1	0.14	0.7151
pH-T	0.00016	0.00016	1	3.18	0.0874
pH-D-T	0.00000	0.00000	1	0.03	0.8634
Error	0.00121	0.00005	24		
Pi	0.00186	0.00093	2	16.56	0.0000
Pi-D	0.00016	0.00008	2	1.45	0.2454
Pi-H	0.00060	0.00030	2	5.34	0.0080
Pi-D-H	0.00059	0.00029	2	5.24	0.0087
Error	0.00269	0.00006	48		

TABLE D-12 (Continued)

Source of Variation	Sum of Squares	Mean of Squares	Degrees of Freedom	F Ratio	Tail Probability
pH-Pi	0.00027	0.00013	2	2.55	0.0890
pH-Pi-D	0.00039	0.00020	2	3.72	0.0313
pH-Pi-T	0.00015	0.00007	2	1.38	0.2603
pH-Pi-T-D	0.00055	0.00027	2	5.19	0.0091
Error	0.00252	0.00005	48		

Pi = Inorganic Phosphate

APPENDIX D

TABLE D-13

TUKEY POST-HOC COMPARISONS OF BASAL, Mg^{2+}-STIMULATED MYOFIBRIL ATPASE ACTIVITIES (pH 7.0: Within and Between Group Differences)

	A_1	A_2	A_3	B_1	B_2	B_3	C_1	C_2	C_3	D_1	D_2	D_3
A_1	-	0.025 **	0.008	0.008			0.001					
A_2		-	0.017 **		0.016 **			0.021 **				
A_3			-			0.010 *			0.008			
B_1				-	0.001	0.010 *				0.010 *		
B_2					-	0.011 *					0.006	
B_3						-						0.001
C_1							-	0.003	0.001	0.003		
C_2								-	0.004		0.001	
C_3									-			0.003
D_1										-	0.005	0.001
D_2											-	0.006
D_3												-

TABLE D-13 (Continued)

Critical Difference

$p < 0.05$: 0.010 μmol $Pi \cdot mg^{-1} \cdot min^{-1}$ (*)
$p < 0.001$: 0.013 μmol $Pi \cdot mg^{-1} \cdot min^{-1}$ (**)

A = SC
B = TC
C = SD
D = TD

1 = 0 mM Pi
2 = 0.10 mM Pi
3 = 1.0 mM Pi

APPENDIX D

TABLE D-14

TUKEY POST-HOC COMPARISONS OF BASAL, Mg^{2+}-STIMULATED MYOFIBRIL ATPASE ACTIVITIES
(pH 6.5: Within and Between Group Differences)

	A_1	A_2	A_3	B_1	B_2	B_3	C_1	C_2	C_3	D_1	D_2	D_3
A_1	-	0.007	0.016**	0.005			0.004					
A_2		-	0.009		0.005			0.001				
A_3			-			0.015**			0.020**			
B_1				-	0.007	0.004				0.007		
B_2					-	0.011*					0.009	
B_3						-						0.002
C_1							-	0.010*	0	0.002		
C_2								-	0.010*		0.003	
C_3									-			0.003
D_1										-	0.005	0.001
D_2											-	0.004
D_3												-

TABLE D-14 (Continued)

Critical Difference

$p < 0.05$: 0.010 μmol $Pi \cdot mg^{-1} \cdot min^{-1}$ (*)
$p < 0.001$: 0.013 μmol $Pi \cdot mg^{-1} \cdot min^{-1}$ (**)

A = SC
B = TC
C = SD
D = TD

1 = 0 mM Pi
2 = 0.10 mM Pi
3 = 1.0 mM Pi